Effect of dietary incorporation of sorghum distillers dried grains with solubles (DDGS) in the hematological and immunological responses of Nile tilapia (*Oreochromis niloticus*), submitted to *Aeromonas hydrophila* challenge.

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Em primeiro lugar quero agradecer aos meus pais por todo o apoio, incentivo e sacrifícios feitos ao longo de todos estes anos de estudo. Sem eles, nada seria possível.

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General Abstract

As part of the master dissertation, an internship was carried between September 19, 2014 and 28 March 2015, at the laboratory of nutrition and health of the fish, Aquanutri, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Botucatu, São Paulo, Brazil. The main research focus of Aquanutri laboratory is the evaluation of the interaction between nutrition and productive performance and health of fish.

This report is divided into two parts. The first part it is described the experimental protocol developed for the production of zebrafish (*Danio rerio*) and scalar (*Pterophyllum scalare*), involving all the developmental stages of these species as reproduction, larvae and juvenile development and maintenance, transport and slaughter. In the second part it is described the experimental study performed to evaluate the effect of the dietary incorporation of sorghum distillers dried grains with solubles (DDGS) in the hematological and immunological responses of Nile tilapia (*Oreochromis niloticus*), submitted to bacterial (*Aeromonas hydrophila*) challenge.

This internship also allowed to obtain complementary knowledge and experience in design and construction of experimental aquatic systems, feed formulation and feed manufacturing. It also allowed to learn different techniques of and chemical analysis, hematological analysis, blood-immunological analysis

Sumário geral

No âmbito da dissertação de mestrado foi realizado um estágio entre 19 de Setembro de 2014 a 28 de Março de 2015, no laboratório de Nutrição e Saúde dos Peixes, Aquanutri, na Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Botucatu, São Paulo, Brasil. Este laboratório tem como principais linhas de investigação a determinação das necessidades nutricionais e o efeito da interação entre a nutrição e o desempenho reprodutivo e a saúde de peixes.

O trabalho de estágio compreendeu duas fases. Na primeira parte é apresentado o plano desenvolvido para a produção de peixe zebra (*Danio rerio*) e de escalares (*Pterophyllum scalare*) envolvendo todas as diferentes fases de produção, desde a reprodução, crescimento larvar e juvenil, manutenção, transporte e abate (trabalho apresentado na Parte I deste relatório).

Durante o período de estágio foi realizado um estudo focado na avaliação do efeito da incorporação de grãos secos de destilaria com solúveis (DDGS) de sorgo nas respostas hematológicas e imunológicas da tilápia do Nilo (*Oreochromis niloticus*), submetidas a um desafio bacteriano por *Aeromonas hydrophila* (trabalho apresentado na Parte II deste relatório).

Este estágio permitiu, também, a aquisição de diversos conhecimentos a nível de delineamento de ensaios experimentais, montagem de sistemas experimentais, maneio de peixes, formulação e produção de alimentos compostos para peixes. Permitiu também aprender técnicas de análises hematológicas, imunológicas e bromatológicas.

Parte I: Estágio

Relatório de estágio no laboratório AquaNutri, UNESP, Botucatu, Brasil

O Laboratório de Nutrição e Saúde de Peixes, AquaNutri, tem como principais linhas de pesquisa a avaliação do valor biológico dos alimentos, a determinação de exigências nutricionais e a avaliação da nutrição e saúde dos organismos aquáticos. A espécie principal utilizada nos estudos é a Tilápia do Nilo (*Oreochromis niloticus*). Para além desta espécie, os alunos são incentivados a trabalhar com outros organismos aquáticos de forma a adquirirem conhecimentos que possam vir a ser necessários em estudos posteriores, como o caso do peixe-zebra (*Danio rerio*) e do peixe escalar (*Pterophyllum scalare*).

A rotina diária do laboratório envolveu a alimentação dos peixes, a confeção de ração, a identificação e remoção de peixes moribundos, a limpeza dos tanques e sistemas de filtragem, acompanhamento de ensaios de crescimento e digestibilidade, manutenção de materiais e dispositivos fundamentais ao funcionamento dos ensaios experimentais. Além disto foi possível aprender métodos de reprodução, manutenção e manipulação de tilápia do Nilo, peixe-zebra e escalar.

Durante o estágio foi ainda possível desenvolver um modelo de produção de peixe-zebra. Este modelo englobou a aquisição de diferentes conhecimentos e de técnicas, nomeadamente de reprodução, de alimentação dos alevins, envolvendo também a produção de alimento vivo, e de manutenção da qualidade da água. A produção de peixe-zebra é um processo relativamente simples, porém meticuloso.

Peixe-zebra, considerações gerais

O peixe-zebra é uma espécie muito utilizada como peixe ornamental e sobretudo como modelo biológico em diversas áreas científicas, tais como toxicologia, evolução, biologia do desenvolvimento, genética, aquacultura, entre outras (Lawrence *et al*., 2012). Contudo, alguns aspetos relacionados com a nutrição comportamento e reprodução do peixe-zebra, ainda não são totalmente compreendidos (Lawrence, 2007 e Lawrence *et al*., 2012).

O peixe-zebra*, Danio rerio* (Hamilton, 1822), é um peixe teleósteo pertencente à família Cyprinidae. Morfologicamente, apresenta um corpo alongado e delgado, com riscas claras e escuras alternadas ao longo do seu corpo (Figura 1), razão pela qual é chamado peixe-zebra (Spence *et al.,* 2008).

Figura 1 - *Danio rerio* (Hamilton, 1822; FishBase 2015)

Esta espécie é nativa algumas regiões do Sul da Asia, caracterizadas por um clima tropical de monção, com estações de chuva e seca pronunciadas que influenciam parâmetros físicos e químicos das águas, bem como a disponibilidade de alimento. O peixe zebra pode ser encontrado em águas paradas com substratos arenosos, em diversos canais de rios ou riachos, e até mesmo em áreas adjacentes de zonas húmidas e campos de arroz, tanto em canais naturais ou em canais e lagoas feitas pelo homem (Spence *et al*., 2008).

O peixe-zebra é um organismo de pequenas dimensões, podendo atingir cinco centímetros de comprimento, e apresenta dimorfismo sexual, as fêmeas geralmente são maior que os machos e têm uma forma arredondada devido à presença de ovos nos ovidutos enquanto os machos possuem um corpo mais delgado e escuro. A sua reprodução é assíncrona, realizando posturas ao longo de vários dias, em períodos irregulares. As fêmeas depositam os ovos no substrato, os quais são posteriormente fertilizados pelos machos. Os progenitores não investem em cuidados parentais. O desenvolvimento dos ovos está dependente das condições físico-químicas do meio, como a temperatura, qualidade da água, entre outras. Após a eclosão, os alevins desenvolvem-se e adquirem movimentos natatórios entre o quarto e sétimo dia (Lawrence, 2007).

Relativamente à dieta, o peixe-zebra é omnívoro. Em ambiente natural alimenta-se de uma grande variedade de crustáceos planctónicos e bentónicos, vermes e alevins de insetos sendo composta (Lawrence, 2007).

Produção de peixe-zebra

O peixe-zebra é tolerante a uma ampla gama de condições ambientais em cultura. A sua adaptabilidade é um reflexo da sua larga distribuição na natureza, estando presentes em diferentes tipos de habitats com propriedades físico-químicas distintas. Contudo é importante salientar que pode existir um custo energético acrescido quando se cultiva fora dos parâmetros ambientais ótimos. Os animais mantidos abaixo das suas condições ótimas necessitam de direcionar mais energia para a manutenção da homeostasia do que para o crescimento, produção de gâmetas e funções imunológicas. Uma diminuição da taxa de crescimento, do número e qualidade da descendência, e sobrevivência, podem ser consequências de condições de cultura sub-ótimas. Desta forma, em sistemas de cultura, é essencial garantir condições físico químicas mais próximas o possível da ótimas (Lawrence, 2007), o que foi tido em conta a quando do desenvolvimento do plano de produção desta espécie no laboratório Aquanutri. Até à data da realização deste estágio, o laboratório Aquanutri nunca tinha produzido esta espécie, razão pela qual foi necessário implementar um modelo de produção construído de raiz. O plano de produção compreendeu diversas fazes desde a fase de reprodução, desova e crescimento dos alevins até o estado adulto.

Reprodução

A produção de peixe-zebra no laboratório Aquanutri foi efetuada em aquários de 40 L e/ou em tanques de 500 L. Cinco dias antes da reprodução, foi preparado o sistema experimental composto por um aquário de 40L, que possuía uma estrutura em rede, que iria receber os reprodutores, um sistema de filtragem (filtro esponja) e aerificação. Este foi devidamente limpo e desinfetado com antifúngico (Figura 2). Depois de cheio, a temperatura da água foi mantida a 28ºC.

A estrutura em rede, onde são colocados os reprodutores, possuía poros mais largos do que os ovos de peixe-zebra, para que estes passassem pela rede e se depositassem no fundo do aquário, de forma a evitar que os reprodutores ingerissem os seus próprios ovos, no momento da desova. A altura da coluna de água nesta estrutura de rede foi ajustada a 1 cm. O fluxo de aerificação do aquário foi reduzido de forma a evitar a resuspensão dos ovos.

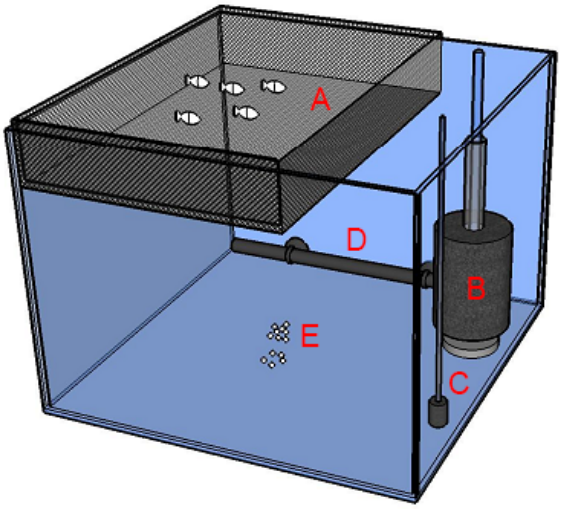

Figura 2 - Representação esquemática do sistema utilizado na reprodução de peixe-zebra. A: estrutura em rede onde são colocados os reprodutores; B: filtro esponja, C: pedra porosa, D: aquecedor, E: ovos de peixe zebra

Depois de preparado o sistema procedeu-se à separação de machos e fêmeas maturados, que foram mantidos em aquários diferentes. Tal procedimento garante uma maior produção de gâmetas femininos e masculinos no momento de reprodução. Tal procedimento evita que as fêmeas estejam constantemente a ovular, devido a feromonas libertadas pelos machos (Spence *et al*., 2008). A separação por sexos foi feita observando diretamente as características morfológicas de ambos os sexos. As fêmeas apresentavam um abdómen mais largo e redondo, e os machos um corpo mais escuro e delgado (Spence *et al*., 2008). Os peixes foram mantidos separados durante 24h.

A reprodução do peixe zebra é regulada sobretudo pelo fotoperíodo. Normalmente, o peixe-zebra desova de madrugada, nas primeiras horas do dia (Lawrence, 2007). Por este motivo, antes de anoitecer os reprodutores foram colocados na estrutura de rede, na proporção de 1 macho para 3 fêmeas, para desovarem. Na madrugada seguinte, os ovos fertilizados, não adesivos e com um diâmetro de 0.7mm acumulavam-se no fundo do aquário (Spence *et al*., 2008). De seguida, a estrutura em rede foi recolhida e os reprodutores foram colocados no aquário de origem. Após a reprodução, caso não haja intenção de obter mais posturas, devemos deixar as fêmeas e os machos juntos, caso contrário, as fêmeas reprodutoras podem morrer, devido à acumulação de ovos nos oviductos (Spence *et al*., 2008). Após a remoção dos progenitores, a altura da coluna de água no tanque foi reduzido para 1 cm, em relação

ao fundo do aquário, de forma a reduzir a pressão hidrostática sobre os ovos. Pouco tempo após a eclosão, os alevins nadam até à superfície da água para encher a bexiga-natatória, pelo que quando mais reduzida for a coluna de água, menos energia será despendida (Lawrence, 2007). Nesta fase, o sistema de filtragem foi desligado, pois pode causar muita movimentação da água, dificultando os movimentos natatórios e alimentação dos alevins.

A reprodução do peixe-zebra em tanques de 500L (Figura 3) foi feita da mesma forma que em aquários de 40L, porém, devido às maiores dimensões, foi possível colocar um número maior de reprodutores, e assim obter posturas com um maior número de alevins.

Figura 3 - Tanques de reprodução do Peixe-zebra. Fotografia tirada por Rafael de Almeida

Manutenção da qualidade da água

O sistema de produção, composto por 19 tanques de 500L, está ligado a um sistema de recirculação de água com filtragem biológica, aerificação e com controlo da temperatura da água a 25ºC. Na Figura 4 é apresentada a constituição do sistema de filtragem, de regulação da temperatura e o sistema de lâmpadas ultravioletas.

Sistema de filtragem

Filtragem mecânica

Detalhe da filtragem mecânica

Filtragem Biológica (argila expandida)

Sistema de aquecimento

Permutador de Calor

Termostato

Bomba e lâmpada de ultravioleta

Figura 4 - Sistema de filtragem e seus detalhes. Fotos tiradas por Pedro Pucci

O sistema de filtragem tem uma capacidade de 18 000L de água. A água que vem dos tanques passa primeiramente por uma zona de filtragem física e, de seguida, por cinco diferentes zonas de filtragem biológica. Por fim a água é canalizada para uma bacia de sedimentação e captada por uma bomba com duas vias. Numa via, a água é encaminhada para permutador de calor a 25ºC e retoma para à primeira zona de filtragem mecânica, na segunda via a água é encaminhada para o sistema de filtragem por luz ultravioleta e posteriormente é direcionada para os sistemas de produção. A manutenção da qualidade da água era monitorizada diariamente, incluindo verificação da temperatura e de pH.

Crescimento dos alevins

Os alevins de peixe-zebra eclodem entre dois a três dias após a fertilização. Após eclodidos, os alevins aderem às paredes do aquário e permanecem imóveis até ao quinto dia de vida, onde começam a encher a bexiga natatória pela ingestão de ar à superfície da água.

Nos dois primeiros dias após a reprodução, os ovos não fertilizados adquiriam uma cor branca opaca, sendo fácil de os distinguir relativamente aos ovos fertilizados transparentes. Os ovos não fertilizados são suscetíveis a contaminações por fungos, os quais podem contaminar ovos embrionados e consequentemente causar mortalidades, por isso são retirados. Os ovos não fertilizados foram recolhidos por sifonação, usando um tubo de diâmetro reduzido, de modo a perturbar o menos possível os ovos embrionados.

Após eclosão dos alevins, o fundo do aquário foi limpo manualmente. Para tal, foi utilizada uma régua para arrastar todos os resíduos acumulados no fundo e finalmente com auxílio de um tubo recolhia-se resíduos por sifonação. Após a recolha dos resíduos, 1/3 da água do aquário foi substituída. Simultaneamente, colocou-se um goblé de 1 Litro com um tubo e um regulador de fluxo de água a gotejar para o aquário para repor o nível da água. A água foi adicionada lentamente para não causar /correntes de água que obriguem os alevins a nadarem mais, e também para manter a temperatura do aquário estável. A acumulação de resíduos no fundo do aquário, além de causar a diminuição da qualidade da água pode levar a altas mortalidades.

Nos primeiros dias de vida, os alevins subsistem principalmente das reservas do saco vitelino até ao início da alimentação exógena, que é coincidente com a conclusão de certas etapas fisiológicas, incluindo o desenvolvimento do sistema digestivo funcional e a capacidade de se movimentarem na coluna de água. Dependendo das condições, esta transição ocorre aproximadamente entre o quinto e sexto dia após a eclosão. Nesta fase, as reservas do saco vitelino esgotam-se completamente. A partir deste ponto, os alevins de peixe-zebra são alimentados com diferentes tipos de alimentos que atendem às necessidades nutricionais, que são mais elevadas nesta fase de vida (Lawrence, 2007).

Alimentação

A seleção apropriada da dieta é uma componente essencial para garantir uma elevada sobrevivência dos alevins. É importante fornecer alimentos que atendam às necessidades nutricionais como também às suas dimensões morfométricas. O peixe-zebra são predadores com uma capacidade de abertura bucal limitada, particularmente durante o estado larvar. No início da alimentação exógena os alevins de peixe-zebra tem uma abertura bucal de aproximadamente 100 µm, por isso, é essencial escolher alimentos com dimensões aproximadas (Lawrence, 2007). Alimentos adequados ao tamanho bucal dos alevins permitem que o alimento seja utilizado mais eficientemente.

Durante o estágio, os alevins foram inicialmente alimentados com gema de ovo cozida, depois com microverme (*Panagrellus redivivus*) e artémia, e finalmente, quando já é possível alimentar-se de partículas de maiores dimensões é fornecida ração.

Gema de Ovo

A gema de ovo é um alimento nutritivo, rico em proteínas (Sartori *et al*., 2009), vulgarmente utilizado por aquariofilistas para alimentar alevins nos primeiros dias de vida. Para preparar gema de ovo, uma gema do ovo é separada da clara e colocada a cozer. Posteriormente a gema cozida é dissolvida em 500ml de água. A solução pode ser armazenada em cuvetes de gelo a -20ºC para uso posterior. Posteriormente, um cubo de gelo da solução de gema de ovo é colocado a diluir em 500ml de água e seguidamente é filtrada numa malha de 60 micrómetros. Esta filtragem permite separar

as partículas de acordo com a sua granulometria, evitando fornecer alimento com dimensões que os peixes possam ingerir e que se acumularia no fundo do aquário.

Dadas as pequenas dimensões dos alevins, nos primeiros dias de vida, é difícil perceber quando estes estão saciados. Por isso, e dado que a gema de ovo cozida reduz consideravelmente a qualidade da água, foram apenas fornecidas pequenas quantidades deste alimento, duas vezes por dia.

Microverme - *Panagrellus redivivus*

A utilização de microverme como alimento vivo para alevins de peixes é uma prática que tem sido usada com sucesso na produção de crustáceos, carpas e peixes ornamentais. Entre vários microvermes, o *Panagrellus redivivus* é um nematode de pequenas dimensões (50 µm de diâmetro) ovovivíparos (Figura 5). O *Panagrellus redivivus* pode ser usado como primeiro alimento dos alevins de peixes por ser relativamente fácil e económico e passível de ser produzido em grandes quantidades. Além disto, é possível manter a cultura destes organismos durante um longo período de tempo, permitindo assim ter sempre uma fonte de alimento vivo disponível para alimentar os alevins de peixes (Christian *et al*., 2004).

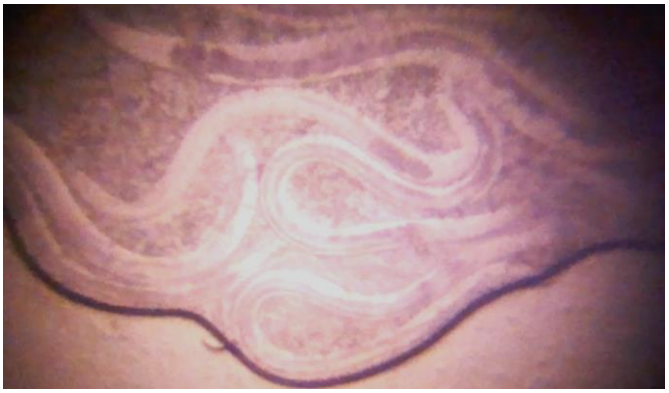

Figura 5 - *Panagrellus redivivus* em meio de cultura, ampliação 10x. (Fotografia tirada por Rafael de Almeida)

Apesar de possuir menos proteína do que a artémia (48% *P. redivivus*, 61% artémia), a sua composição aminoacídica é semelhante à da artémia com um conteúdo lipídico (25%) significativamente superior ao da artémia (17%) (FAO, 1996). A qualidade destes nematodes pode ser influenciada pelo meio de cultura utilizado, sendo assim possível modificar a composição química dos nematodes (Schlechtriem *et al*., 2004).

Para cultivar *Panagrellus redivivus* foi utilizado um inóculo inicial, um meio de cultura e um recipiente raso, opaco com tampa. O meio de cultura foi composto por aveia (farinha ou flocos), ao qual se adicionou água até formar uma pasta com a consistência do mel. É importante conseguir uma consistência adequada para que os microvermes se possam movimentar livremente pelo meio de cultura. Caso o meio ficasse pouco consistente era adicionada mais aveia. Uma vez que os microvermes tem a facilidade de absorver substâncias presentes no meio, o meio de cultura foi enriquecido com uma pré-mistura de minerais e vitaminas. Posteriormente toda a mistura foi igualmente distribuída pelo fundo do recipiente de forma a perfazer 1 cm de altura. Finalmente foi adicionado o inóculo de microverme e o recipiente foi mantido fechado (para evitar a contaminação por outros organismos), num local escuro, numa temperatura de 25ºC (Figura 6). Uma vez por dia, o recipiente foi aberto de forma a renovar o ar da cultura.

Figura 6 - Recipiente com meio de cultura de microverme. (Fotografia tirada por Rafael de Almeida)

Dois dias após a inoculação foi possível observar o movimento dos microvermes na superfície do meio. Após o terceiro dia, os microvermes, que sobem pelas laterais do recipiente, são recolhidos com auxílio de um pincel, e posteriormente fornecidos aos alevins de peixe-zebra, tendo o cuidado de não colocar o pincel dentro da cultura de microvermes de modo a não contaminar a água dos alevins. Caso a cultura de microverme ficasse seca, adiciona-se um pouco de água. De forma a garantir sempre o *stock* de microverme, são produzidas várias culturas diferentes. As culturas deixam de ser viáveis quando adquirem uma coloração escura, um cheiro forte, e os microvermes deixam de subir pelas paredes do recipiente.

Cultura de Artémia

Para obter nauplios de artémia, quistos comerciais foram incubados em água salgada, a uma temperatura de 25ºC durante 24 horas sobre luz artificial, numa estrutura cônica e devidamente aerificada.

Devido ao elevado custo dos ovos de artémia, a utilização de microvermes nos primeiros dias dos alevins, torna-se uma mais-valia económica, permitindo reduzir os custos de produção. É possível também fornecer microvermes e náuplios de artémia alternadamente. Contudo, a partir de certo ponto do crescimento, os alevins deixam de mostrar interesse nos microvermes e passam a preferir alimento maior, como artémia ou mesmo ração de granulometria muito pequena. Este ponto é determinado com base na observação do comportamento dos alevins quando lhes é fornecido o microverme. Se os alevins não procurarem ingerir o microverme fornecido, a dieta é alterada, passando-se a fornecer náuplios de artémia. A quantidade de produção e fornecimento de artémia é ajustada com base na quantidade de alevins que se pretende alimentar.

Ração

Quando os peixes adquirem a capacidade de ingerir alimento maior do que os náuplios de artémia, estes são substituída por ração comercial. Neste momento estão a ser desenvolvidos e estados diferentes alimentos experimentais desenvolvidos pelo próprio laboratório. Infelizmente devido à curta duração do estágio, não foi possível acompanhe este ensaio até ao fim. A título de exemplo é apresentada a composição das dietas experimentais (Tabela 1).

Manutenção

Quando os alevins atingiram o estado adulto, os peixes foram transferidos para tanques ou aquários maiores, onde foram alimentados com ração duas vezes por dia até a saciedade visual. Posteriormente foram utlizados para realizar ensaios experimentais ou transportados para outras instalações para outras finalidades.

Tabela 1 - Ingredientes e composição nutricional das rações formuladas para peixe-zebra.

Ingredientes (% Peso seco)					
Ração	**PURIFICADA**	**SEMI-PURIF**	**VEGETAL**	**MISTURA**	**ANIMAL**
Gelatina	5.00	5.00	0.00	0.00	0.00
Albumina	45.92	37.37	0.00	0.00	0.00
Amido	36.96	35.00	0.00	0.00	0.00
Soja. óleo	2.78	3.92	0.95	1.13	0.75
Fosfato bicálcico	5.39	4.63	4.62	2.85	1.32
Celulose	3.02	3.15	0.00	1.75	1.75
Farinha de salmão	0.00	10.00	5.00	30.00	35.00
Farelo de trigo	0.00	0.00	6.61	5.00	10.00
Milho grão	0.00	0.00	10.00	14.82	18.59
Quirera de arroz	0.00	0.00	5.00	10.00	10.00
Bagaço de soja	0.00	0.00	45.63	23.00	0.00
Glúten de milho	0.00	0.00	6.00	10.39	0.00
Concentrado p. soja	0.00	0.00	15.00	0.00	0.00
Farinha de vísceras	0.00	0.00	0.00	0.00	21.58
DL-metionina	0.00	0.00	0.20	0.00	0.00
L-Treonina	0.00	0.00	0.06	0.04	0.00
L-Triptofano	0.00	0.00	0.00	0.09	0.08
FIXOS					
BHT	0.02	0.02	0.02	0.02	0.02
Vit. C	0.18	0.18	0.18	0.18	0.18
NaCl	0.10	0.10	0.10	0.10	0.10
Cloreto de Colina	0.13	0.13	0.13	0.13	0.13
Premix Vit/min	0.50	0.50	0.50	0.50	0.50
TOTAL (%)	**100**	**100**	**100**	**100**	**100**

Composição proximal (% Peso seco)					
Ração	**PURIFICADA**	**SEMI-PURIF**	**VEGETAL**	**MIX**	**ANIMAL**
EB (kJ/g)	17.2	17.21	17.0	16.9	17.1
PB (%)	40.0	40.0	40.0	40.0	40.0
FB (%)	3.01	3.00	4.07	3.89	3.01
EE (%)	3.00	5.10	3.88	7.06	10.89
AMIDO (%)	32.38	30.66	21.76	26.24	27.62
Lys (%)	2.46	2.56	2.41	2.47	3.03
Met (%)	1.40	1.31	0.74	0.76	0.90
Thr (%)	1.87	1.81	1.50	1.50	1.58
Trp (%)	0.46	0.40	0.40	0.30	0.30
Ca (%)	1.37	1.58	1.52	2.00	2.51
P total (%)	1.22	1.32	1.58	1.73	1.92
P disp (%)	1.07	1.07	1.07	1.07	1.07

Transporte

Para o transporte do peixe-zebra, foram utilizados sacos de transporte de 20 L de capacidade, com bordas redondas. Trezentos peixes eram transferidos para os sacos preenchidos com metade do volume de água, e posteriormente era injetado ar comprimido. Finalmente cada saco foi selado com um nó e fitas de borracha, para evitar fugas de ar. Este método permite transportar os peixes seguramente durante aproximadamente 2 horas, a uma temperatura de 25ºC.

Escalar

Durante o estágio foi também possível acompanhar e participar na produção do escalar.

O *Pterophyllum scalare* (Schultze, 1823), vulgarmente chamado por escalar em Portugal, e peixe acará-bandeira no Brasil, pertence à família dos Ciclídeos Amazónicos (Yamamoto *et al.*, 1999). O escalar tolera um pH entre 6.0 a 8.0 e a temperatura ideal é de 24ºC a 30ºC. Morfologicamente caracteriza-se por ter um corpo em forma de disco com uma barbatana dorsal e anal de grandes dimensões e podem atingir 8 cm de comprimento. (Figura 7). Devido à sua grande popularidade como peixe ornamental existem muitas preocupações sobre a recolha de indivíduos do ecossistema natural para exportação, o que acarreta elevado impacto nos *stocks* naturais. Assim, um melhor conhecimento do seu sistema de reprodução, poderá ser uma ajuda no desenvolvimento de sistemas de cultivo deste peixe, reduzindo e mesmo evitando a sua captura em meio natural (Yamamoto *et al*., 1999).

Figura 7 - *Pterophyllum scalare* (Schultze, 1823) Fotografia tirada por Rafael de Almeida.

Durante a época de reprodução, os machos de escalar estabelecem territórios em espaços com muita vegetação aquática. Seguidamente os machos tentam cortejar as fêmeas livres, e caso alguma corte seja bem-sucedida, a fêmea liberta ovos que posteriormente são fertilizados pelo macho. As fêmeas depositam os ovos na vegetação aquática, onde posteriormente são fertilizados pelos machos. Depois da fertilização, os progenitores dedicam-se à sua proteção, limpeza e ventilação. Após a eclosão dos ovos (48 horas depois), os alevins permanecem fixos à superfície da vegetação através de uma glândula específica presente na cabeça e continuam a receber cuidados parentais. Perante algum perigo, os progenitores guardam os alevins na cavidade bucal para as proteger. Os machos são mais ativos na defesa das crias do que as fêmeas (Yamamoto *et al*., 1999).

Produção de Escalar

O escalar é uma espécie que forma casais para reproduzir, por isso, não é possível obter posturas juntando simplesmente um macho com uma fêmea. É necessário primeiramente identificar casais compatíveis. A forma ideal para conseguir identificar casais compatíveis, passa por colocar vários indivíduos, machos e fêmeas, num aquário e tentar identificar um casal por observação comportamental. Quando os escalares formam um casal, normalmente, costumam isolar-se dos restantes indivíduos do aquário e andam sempre juntos. Após identificado é crucial conseguir separar o casal dos restantes indivíduos e colocar num aquário separado, com filtragem, aerificação e a temperatura controlada (25ºC)

Em ambiente de produção, certos tipos de práticas de cultivo e fatores de *stress*, podem levar a que os escalares eliminem toda a sua ninhada. A simples aproximação do tratador ao aquário, onde se encontra um casal com a postura, pode representar um estímulo negativo para os progenitores, levando-os a eliminar todos os alevins. Desta forma, torna-se mais seguro logo após a fertilização dos ovos separar os ovos dos progenitores.

Para separar os ovos dos progenitores é inserida uma calha de plástico rugoso dentro do aquário, antes da postura. Seguidamente retira-se qualquer tipo de ornamentação ou vegetação que esteja dentro do aquário. Desta forma, a fêmea apenas pode depositar os ovos na calha de plástico (Figura 8).

Figura 8 - Postura de escalar numa calha de pvc. Fotografia tirada por Rafael de Almeida

Após 24 horas da deposição dos ovos, a calha de pvc é recolhida e colocada dentro de um gobelé, com água do aquário, devidamente aerificada. De seguida, o gobelé é colocado dentro de outro aquário, de forma a manter a temperatura da água estável (25ºC), e colocado antifúngico na quantidade indicada pelo fornecedor. Após a eclosão, os alevins libertam-se da superfície e adquirem natação livre. Nesta fase, os alevins são colocados num aquário com uma coluna de água de 2 a 3cm.

Nos primeiros dias de vida, embora os alevins ainda possuam reservas vitelinas, é fornecida uma pequena quantidade de gema de ovo cozida. Posteriormente, acompanhando o crescimento morfológico, é fornecido sequencialmente microverme, náuplios de artémia e ração tal como na produção de peixe-zebra. Devido às maiores

dimensões do peixe escalar do que a do peixe-zebra, é possível iniciar a alimentação com microverme logo nos primeiros dias de vida.

O plano de maneio adotado durante a fase de alevins do peixe escalar é semelhante ao do peixe-zebra, evitando o uso de forte aerificação, que possa causar correntes forte, a ajustando a quantidade de alimento a fornecer de modo a garantir a manutenção da qualidade da água, uma vez que a rápida redução da qualidade da água pode causar a morte de toda a cultura. Ao longo do crescimento dos alevins, a altura da coluna de água é aumentada gradualmente, disponibilizando mais espaço e maiores quantidade de oxigénio. Este aumento gradual, permite que o alevim economize energia na procura de alimento, refletindo-se uma maior taxa de crescimento. Um terço da água é substituído diariamente por água à mesma temperatura, e a limpeza do aquário é feita como descrito na produção de peixe-zebra.

Os alevins de *P.scalare* são mais resistentes do que os alevins de *Danio rerio* a condições adversas, contudo é necessário estar atento a qualquer problema iminente.

Conclusões

O estágio no laboratório Aquanutri possibilitou a aquisição de vários conhecimentos relacionados com a produção e manutenção do peixe zebra e do escalar em condições controladas. A familiarização com técnicas de produção de peixes facilitou um melhor entendimento do funcionamento dos sistemas de reprodução, crescimento e manutenção do peixe-zebra e escalar.

Este estágio proporcionou o desenvolvimento do espirito crítico e criatividade, essencial no desenvolvimento e aperfeiçoamento das técnicas de produção e manutenção da saúde dos peixes.

A aquisição destes conhecimentos poderá revelar-se importante no futuro.

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Parte II: Experimental trial

Effect of dietary incorporation of sorghum distillers dried grains with solubles (DDGS) in the hematological and immunological responses of Nile tilapia (*Oreochromis niloticus*), submitted to *Aeromonas hydrophila* challenge

Abstract of the experimental study

Soybean meal (SBM) is currently the most commonly used plant protein source used in fish feeds, however, its replacement by ingredients not directly suitable for human consumption, are required. Distillers dried grains with solubles (DDGS) is a by-product of dry grind fuel ethanol production. Comparatively to the most common DDGS, the corn DDGS, sorghum DDGS (SDDGS) is slightly higher in crude protein, significantly higher in acid detergent fiber, ash and tryptophan, and lower in crude lipids, lysine and arginine being necessary to fully study the repercussion of SDDGS not only on growth performance and feed utilization but also on the general heath and immune status of fish. Therefore, a study was conducted to determine the effect of increasing dietary levels of SDDGS on resistance, hematological and immune response of Nile tilapia, *Oreochromis niloticus*, subjected to *Aeromonas hydrophila* challenge. Five diets were formulated containing 0 (control), 10, 30, 50 and 70 % of SDDGS as partial replacement of soybean and corn meals. Each diet was fed to juvenile Nile tilapia (32.8 ± 1.0 g) in quadruplicates tanks, four times per day, to apparent satiation for 90 days. After this period, blood samples were collected from twenty fish, four per diet. Then, fifty fish, ten per diet, were submitted to a bacterial challenge by *Aeromonas hydrophila*. Mortality was recorded during 15 days. Post challenge blood samples were collected for the evaluation of hematological and immunological responses.

Before challenge, dietary inclusion of SDDGS had no significant effect on the hematological and immune parameters. After challenge, tilapia fed the diets incorporation 30 to 50% of SDDGS had a higher, even though not statistically significant, resistance against *Aeromonas hydrophila*. After the bacterial challenge, it was verified that SDDGS incorporation lead to an increase of total number of neutrophils, plasma albumin, albumin/globulin ratio and lysozyme activity, although not statically different, evidencing a possible up-regulation of the innate immune response due to the dietary incorporation of the SDDGS. Additionally, and in contrast with other dietary treatments, the SDDGS 50% diet was the only treatment where no fish died in first five days of bacterial challenge.

Together, these results suggest that, even the highest incorporation level of SDDGS did not compromise the bacterial resistance of Nile tilapia and the SDDGS may, inclusively enhance the tilapia resistance against *Aeromonas hydrophila* through an up-regulate innate immune response. Further research, are need to corroborate the trend observed in this trial, including supplementary immunological tests.

Key words: Alternative protein sources, distillers dried grains with solubles, diseases resistance; hematology; immune response

Introduction

1. Aquaculture

1.1. World aquaculture

As global population continues to increase demand for aquatic food products is also rising at a high rate. For this reason aquaculture is currently the fastest growing food-producing sector in the world (Subasinghe *et al.*, 2009, FAO, 2014).

According to the up-to-date statistics by FAO (FAO, 2014), world aquaculture production reached a record of 90.4 million tons (live weight) in 2012 (US $ 144.4 billion), including 66.6 million of tons of marine organisms except algae (US $ 137.7 billion). In 2012, aquaculture production contributed to about 45% of a total of 158 million tons to world fish production (Figure 1; FAO, 2014).

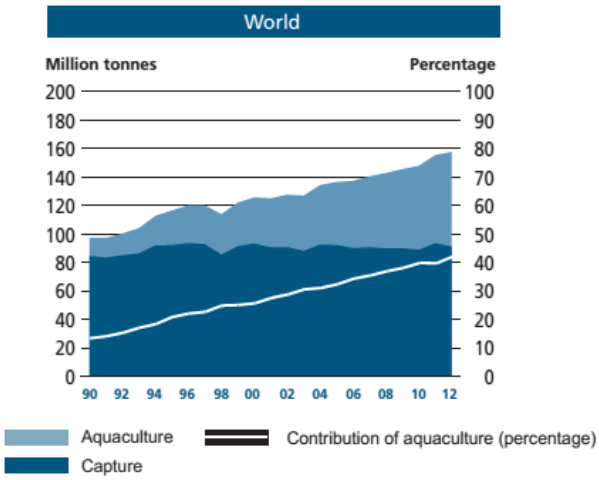


Figure 1 - Total world capture fisheries and aquaculture production in 2012 (FAO 2014).

Asian countries are the main producers of aquatic organisms, contributing with about 88% of total volume in aquaculture. It is estimated that more than 600 aquatic species, including algae, molluscs, crustaceans and fish, are produced worldwide in various farming systems, with different densities and technological systems (extensive, semi-intensive and intensive), in saltwater, freshwater or brackish (FAO, 2014).

1.2. Aquaculture in Brazil

In recent years, Brazilian aquaculture has undergone many transformations, making it an activity with great potential for the country's industry. Brazil is the second

largest aquaculture producer in the Latin American and Caribbean region, with rapid growth of farmed fish production, increasing from 172 000 tonnes in 2000 to 629 300 tonnes in 2011 (FAO 2010).

This activity is of great importance in the food supply of local communities and in international market, exporting products to countries here aquaculture is insufficient or nonexistent (Sanches el al, 2013). In 2011, aquaculture contributed to about 44%, up from 21% in 2000 (1.05 million tons), for the total fish production in Brazil (FAO 2010). It is estimated that by 2015, this contribution will increase to about 47% (1.78 million tons) (Capobianco, 2013). Freshwater aquaculture accounted for 87% (545 300 tonnes) of total aquaculture production in 2011, dominated by the production of tilapia, carps and some indigenous species, including catfish of the Pimelodidae family; the “curimatãs” and “jaraquis” (family Curimatidae); “round” fishes (“tambaquis”, “pacus” and “pirapitingas”) of the Characidae family; “tucunarés” of the Cichlidae family (FAO 2010).

The aquaculture production of tilapia was introduced and spread throughout the Brazilian territory in the 50s, however, tilapia was a little-prized species until the mid-90s. Even thought, today is the main species cultivated in Brazil, with 155 450 tons in 2010. According to official data, between 2000 and 2010 the production of tilapia grew on average 17% a year, more than the average growth of 10 % recorded by the aquaculture (Figure 2; Kubitza *et al.*, 2012) .

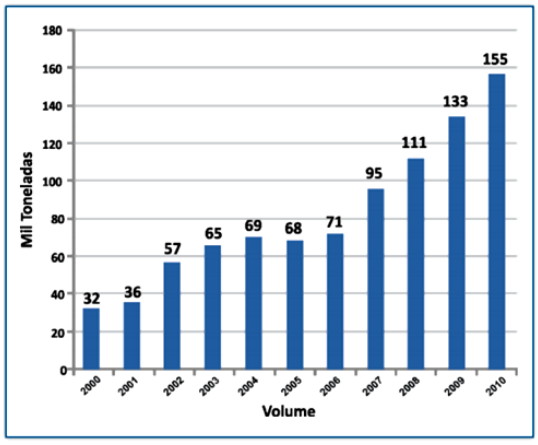


Figure 2 - Official data of tilapia production in Brazil. Source:(Kubitza *et al*., 2012).

Production of tilapia in Brazil corresponds to 47% of total aquaculture fish production of the country, followed by the production of tambaqui, tambacu, carp and pacu (Capobianco, 2013). The optimal environmental conditions, in addition with the

abundance of water resources in Brazil provide excellent conditions for tilapia aquaculture (Fitzsimmons, 2000).

1.3. Nile tilapia

The Nile tilapia, *Oreochromis niloticus* (Linnaeus , 1758) (Figure 3), belongs to the *Cichlidae* family is endemic of Nile River in Africa but it is widespread in tropical and subtropical wild regions around the world (Ayroza, 2009). It occurs in a variety of freshwater habitats such as rivers, lakes and irrigation channels. Nile tilapia is a diurnal species that mainly feeds on phytoplankton and zooplankton. Nile tilapia reproduces throughout the year by oviparity and after hatching, the larvae are incubated by the parent in the oral cavity (Mouthbrooder). This species tolerates a temperature ranging from 8 ° C to 42 ° C, but the natural temperature is between 13.5 ºC and 33°C (FishBase).

Figure 3 - Nile tilapia (Oreochromis niloticus Linnaeus , 1758) Photography by Rafael de Almeida

Nile tilapia has an excellent growth rate, a huge adaptability to a wide range of physical and environmental conditions, ability to reproduce in captivity, high disease resistance, good meat quality and low trophic level (Watanabe *et al*., 2002).

Nile tilapia is adapt to complete commercial diets based on animal and plant protein sources. This species can tolerate higher dietary fiber (3.5%) and carbohydrate concentrations (30-40%) than most of the other aquaculture fish species. The protein requirement of tilapia decreases with age, being higher during the fry (45 -41%) and juvenile (40 – 36%) stages and lower (35 -30%) for larger tilapia (Davis *et al*., 2009).

Freshwater fish species such as tilapia, carp and catfish have a relatively low requirements for methionine, this results in an advantage in the way that it makes it simpler to formulate diets with alternative ingredients in comparison with other species. This seems to give species such as tilapia the upper hand in the matter of substitute fish

meal with a less expensive protein source. Besides, various studies suggest that a low level of fish meal in fry and fingerlings feed proved to be an advantage. In terms of dietary protein sources there is considerable information regarding this subject. Concerning alternative protein for tilapia it includes fishery by-products, terrestrial animal by-products, oilseed plants, aquatic plants, single-cell proteins, grain legumes, plant protein concentrates and cereal by-products (Davis *et al.,* 2009)

All these qualities contribute to the great success of tilapia aquaculture production worldwide, being the third most important fish in aquaculture after carp and salmon. Nile tilapia is produced in a wide variety of production systems in both fresh water and brackish water, either in temperate, subtropical or tropical climates. This fish species has been recognized as the “elected” species for intensive aquaculture production, and it is on the path of becoming the most important of all cultured fish in the world (Lim *et al.*, 2011)

2.1. Alternative feed ingredients

Even thought, tilapia are among the easiest and most profitable fish produced in aquaculture, partially due to their low trophic level (omnivorous diet), feed cost still represents the highest expense in its production (up to 50% of total cost production; Lim *et al*., 2011). Protein fraction is usually the most expensive component of the diet (Coyle *et al*., 2004), traditionally provided by the incorporation of fish meal. However, due to economic, environmental and social constrains, fish meal has been replaced by more economical protein source, as soybean meal, in order to improve cost-effectiveness of feeds. Soybean meal is less expensive than fish meal, has a good nutritional value with high crude protein content and a reasonably balanced amino acid profile and high availability (Gatlin *et al*., 2007). It presents some disadvantages compared to fish meal such as lower levels of protein (amino acids, particularly lysine, methionine and threonine), of digestible energy, of available phosphorus and presence of some anti-nutritional factors (Gatlin *et al*., 2007; Furuya and Furuya, 2010).

Soybean meal is considered to be the most nutritious plant protein source, being one of the most used ingredient in aquafeeds. However, due to the “food-feed competition” and rising prices of conventional feed ingredients, such soybean meal, wheat and corn meals, innovative feed ingredients, not directly suitable for human consumption, are required.

An alternative protein source should have certain characteristics, such as good availability in the market, competitive prices, and the ability to be easy to handle, transport and store. From a nutritional point of view, alternative protein sources to the fish meal must possess some essential features, since aquaculture fish require higher protein levels than other animals bred for human consumption. Thus, this ingredient must contain a moderate / high protein, with a favorable amino acid profile, good digestibility, good palatability and low in fiber and anti-nutritional factors (Gatlin *et al*., 2007).

The search for alternative aquafeed ingredients, nutritionally adequate, safe and sustainable has therefore come to the forefront. Plant ingredients other than soybean meal or other oleaginous plants, particularly plant by-products from food, fermentation, and pharmaceutical industries, are promising ingredients. Dried distiller´s grain with solubles is an alternative protein source with increased interest for aquafeeds.

2.2. Distillers dried grains with solubles (DDGS)

In the last decades, concerns about greenhouses gases, shortage and dependency on petroleum-based fuel have led to increased production of ethanol. The ethanol industry use a method that evolves yeast fermentation and distillation of cereal grains (corn, wheat, barley or sorghum), obtaining ethanol, carbon dioxide and the residue named distillers dried grains with solubles (DDGS). DDGS are a concentrated source of nonfermentable components from the original grain and are relatively high in protein, fiber, lipid, and ash. In fish diets, the use of DDGS is still limited, however, recent research have shown that DDGS is a potential alternative ingredient for omnivores fish, such as tilapia (Lim *et al*., 2011; Schaeffer *et al*., 2011). This co-product is readily available and are less expensive than most conventional plant ingredients, such as soybean meal and corn on a protein-cost basis (Welker *et al.*, 2014a).

Due to increased demand of low-cost protein sources such as DDGS, the market for this product has increased considerably, especially in the United States. Currently, United States is by far the largest producer of grain-based ethanol in world as well as the major exporter country, shipping for Europe, South-America and Asia (Jensen and Björnsson, 2012). After the United States, Brazil is the second largest world producer of ethanol, contributing around 25 % to the global production. Most of the ethanol produced in Brazil is obtained from maize and sorghum grains (Figure 4). Estimates indicate that

the global ethanol production will increase in 70% until 2022, and Brazil will continued to be one of the major producers countries (Oecd-Fao, 2013).

Figure 4 - Sorghum grains. Photo from www.Shutterstock.com

The quality and nutritional composition of DDGS varies depending on many aspects, such as the source and quality of grain, fermentation process, including time, efficiency of drying process and the amount of distillers solubles added. Typically, the protein content of DDGS is moderately high, reaching 250–450 g/kg depending on the grain source. Corn DDGS have a protein content approximately of 28%, 10 % of lipid and 11% of crude fibre. Sorghum DDGS (Figure 5) is slightly higher in crude protein (34%), lower in crude fiber (9.7%) and lipid (9.8%) compared to corn DDGS (Table 1) (Welker *et al.,* 2014a).

Figure 5 - Sorghum DDGS. Photo from www.indiamart.com

For the correct evaluation of a new ingredients in aquafeeds, it is crucial not only to evaluate its effect on growth performance and feed utilization, but also to address its modulation action on well-being, immune status and general animal health (Stein and Shurson, 2009; Oliva-Teles, 2012). This is the case of DDGS. Due to the presence in this feedstuff of yeast used for the ethanol production process, as well as the NSP (non-starch polysaccharide) fraction, DDGS may have probiotic/prebiotic effect that deserves to be explored. In fact, yeast cells have many biologically active compounds with

potential immunological effects, including β-glucans, mannan-oligosaccharides, chitin, nucleotides, and glutamate. Also, oligosaccharides present in the NSP fraction may have a prebiotic effect, thus modulating gut microbiota community and also having immunological properties. Dietary incorporation of DDGS may contribute to boost the fish immune status and diseases resistance, giving an extra value to this ingredient. However, from another perspective, DDGS may also compromise the intestinal immune status and physiology and so it may affect disease resistance.

Table 1 - Proximate composition (g/kg dry matter) for soybean meal (SBM) and DDGS from corn and sorghum. Average values are given with the range, in parentheses. Adapted from (Welker *et al*., 2014a).

	SBM	DDGS source	
		Corn	Sorghum
Dry matter	900	910 (870-920)	890 (880-900)
Crude protein	450	280 (260-310)	340 (301-390)
Fat	5	100 (90-117)	98 (77-117)
Crude fiber	70	110 (102-130)	97 (72-124)
Ash	61	52 (45-67)	42 (33-50)
Phosphorus	6	9 (7-10)	7 (4-9)

3. Nutrition and fish health

Aquaculture has become an increasingly industrialized activity with a continuous expansion around the world. Associated with the rapid growth, certain issues arise about possible negatives effects caused by intensive production, which might affect the sustainable development of aquaculture (Barros *et al*., 2014). In this production conditions, fish are exposed to stressful factors, such as confinement, poor environmental conditions and handling husbandry. All these stressful factors can influence the immune system of fish, well-being and health status, leading to an increase in diseases problems, resulting in major economic loss to aquaculture (El-Boshy *et al*., 2010). As an example, in Brazil, due to rapid expansion and industrialization, Nile tilapia farming has been negatively affected by infection diseases, causing significant economic loss and presenting a major constraint to its expansion (Barros *et al*., 2014).

Nutrition and feeding play a critical role in fish health and disease outbreaks, mainly within intensive aquaculture systems. Indeed, there is a close relationship between nutrient imbalance and the presence of toxic and antinutritional factors in feed

ingredients on the general health condition. Under stressful conditions, fish activate biological defense mechanisms, to recover the equilibrium, which are supported by endogenous sources of nutrients (Kiron, 2012; Machado, 2014). Therefore, proper nutrition plays an important role in the normal growth of aquatic organisms as well as the provision of basic nutrient requirements for immune system. Nutritional strategies can be developed to enhance the fish immune system with the aim of increasing resistance to infectious diseases and thus avoid economic loss (Pohlenz and Gatlin, 2014).

Unbalanced diets, with an inadequate nutritional content, can lead to nutritional deficiencies, compromising the performance and health of animals. For instance, protein and amino acids deficiencies can prejudice immune functions and increase the susceptibility to infection diseases, since protein malnutrition reduces the AA protein concentration in plasma that play an important role in the immune response (Oliva-Teles, 2012; Machado *et al*., 2015). On the other hand, a proper nutrition, fortified with all the essential nutrients (amino acids, essential fatty acids, vitamins or minerals) can enhanced fish health and resistance to diseases. In addition to essential nutrients, other functional constituent's, like prebiotics, probiotics and immunostimulants are currently being considered in fish nutrition aiming to improve fish growth, health, tolerance and resistance do diseases (Oliva-Teles, 2012).

In an attempt to mitigate the effects caused by diseases in aquaculture and reduce the economic losses, multiple preventive and therapeutic methods have been developed, including the use of functional constituents (Newaj-Fyzul and Austin, 2014). Approaches like the use of antimicrobial compounds, prebiotics, probiotics, vaccines, dietary supplements and immunostimulants can be used to improved fish defenses against stress factors and pathogens invasions (Newaj-Fyzul and Austin, 2014).

Immunostimulants are natural or synthetic compounds that can reinforce an immunocompromised fish system promoting a non-specific immune response, antibody production and/or increase the inflammatory response (Newaj-Fyzul and Austin, 2014). The use of immunostimulants in fish feed may be a strategy not only to improve the defense response against pathogens, but also to find alternatives to antibiotics and chemotherapeutic used to treat diseases in fish. Natural Immunostimulants are biocompatible, biodegradable and safe to the environment and human health (El-Boshy *et al*., 2010).

Use of β-glucans has been reported as a natural immunostimulants with enhancing resistance to specific diseases, including aeromonadiasis, enteric redmouth,

Hitra disease, pasteurellosis and vibriosis (Newaj-Fyzul and Austin, 2014; Dalmo and Bøgwald, 2008). This polysaccharide is present in the cell wall of manifold organisms, like plants, fungi, bacteria, algae and yeast, in different morphological forms and immunomodulatory capabilities (El-Boshy *et al*., 2010; Barros *et al*., 2014). β-glucans act by stimulating the nonspecific and cellular immune parameters, increasing leucocyte cells, especially the neutrophils and monocytes populations. Studies have reported that the use β-1.3 glucans led to an increase of lysozyme and serum complement levels, phagocytic and respiratory burst activity.

In the particular case of new ingredients for aquafeed, its composition may represent a challenge. Some characteristics of DDGS, like its high fiber content, phytic acid, and digestibility, may interfere with gut function, welfare and health of fish being, therefore, necessary to evaluate if high inclusion levels of this ingredient may compromise general health of fish. On the other hand, DDGS contains substantial amounts of yeast *Saccharomyces cerevisiae*, which may have some beneficial characteristics*.* The dietary inclusion of yeast has been associated to beneficial effects in terms of digestive capacity (Castro *et al*., 2013) as well as in terms of innate immune system response and resistance against pathogens (Welker *et al*., 2007; Oliva-Teles, 2012; Salvador *et al*., 2012). According to Barros *et al*., (2014) yeast may increase the the concentration of lysozyme and complement system components in plasma that enhance the phagocytic activity of macrophages. Under this context, different studies have examined the effect of the DDGS on the immune response and resistance of fish to bacterial diseases. Lim *et al*. (2009) and Li *et al.* (2012) observed that inclusion of DDGS in the diets significantly improved resistance to *Edwardsiella ictaluri* infection. In contrast, other studies conducted with Nile tilapia using diets containing various levels of DDGS from different sources (Lim *et al*., 2007; Shelby *et al*., 2008; Li *et al*., 2011; Welker *et al*., 2014b) did not result in improved innate immune responses and resistance to *Streptococcus iniae.*

3.1 Fish Disease (*Aeromonas hydrophila*)

Around the world, aquaculture producers face huge economic losses due to high mortalities caused by bacterial diseases. The *Aeromonas hydrophila*, an etiological agent of several diseases with a worldwide distribution, is one of major bacteria that cause mortality in several species, including Nile Tilapia (Abdel *et al*., 2008). This pathogen is a gram-negative movable rod-shaped bacterium which is commonly isolated from fresh water ponds and which is a normal inhabitant of the gastrointestinal tract (Swann and White, 1991). However, under the stressful conditions of intensive aquaculture production (high densities, high ammonia, low dissolved oxygen, handling and co-infection), this bacterium adopt an opportunistic behavior, being capable of causing disease outbreaks (Lee *et al*., 2015). An infection caused by *Aeromonas hydrophila* can have many different symptoms. Fish can be sudden death or otherwise suffer from lack of appetite, swimming abnormalities, abdominal bloating, exophthalmia, local hemorrhage, pale gills and skin ulcerations. Internally, there may be accumulation of ascetic fluid, anemia, and damage to the liver and kidneys (Austin and Austin, 2007). Symptoms will vary depending on the virulence of the organism, the presence or absence of bacteremia or septicemia, stress factors and the fish resistance to infection. (Swann and White, 1991; Garcia and Moraes, 2009). Outbreaks of *Aeromonas hydrophila*, as well as other pathogens, can be prevented by promoting proper handling practices, stocking densities, water quality and nutrition. However, in case of infection, fish rely on its non-specific immune system (also called innate immunity) to fight against pathogens (Swann and White, 1991; Uribe *et al*., 2011).

3.2. The Fish immune system

Like vertebrates animals, fish have the capacity to recognize and respond defensively against harmful living and non-living agents, such ability is called Immunity. Therefore the awareness of foreign agent's result in a well integrate response between immune cells, molecules and memory this process is called the immune system.

Classically, the immune system is divided into two parts, characterized by being different in speed and specificity. The nonspecific (innate) immune system is the first line of defense against any invader, at any temperature. After that, the specific defense proceeds, taking time to act depending on temperature (Ellis, 2001; Uribe eta l., 2011).

This innate response consists in a combination of physical barriers, cellular and humoral responses (Lee *et al.,* 2015). Since fish live in an environment full of potentially harmful agents, physical barriers, like skin, gills and gut mucosal, are the first line of defense mechanism, providing physical and chemical protection, with aid of cellular and humoral components (Kiron, 2012). Cellular responses comprises a series of mechanisms that focus on pathogen kill, mainly by phagocytosis. Humoral response includes a series of cellular receptors and molecules that are soluble in plasma and body fluids, able to destroy invading agents (Uribe *et al.,* 2011). Cellular defenses of fish involved leukocyte such as lymphocytes, monocytes (macrophages) and neutrophils (granulocytes) (Iwama and Nakanishi, 1996). Monocytes are one of the most important cells in fish immune response, playing a crucial role in inflammatory processes, cytokines production, phagocytose and kill of pathogens immediately after their recognition (Lee *et al.,* 2015). Neutrophils also participate in inflammation processes and are responsible for cytokines production, phagocytose and recruitment of immune cells to the damage or/and infected area. Therefore, the immune functions of this cells can vary among different fish species (Lee *et al.,* 2015). The activity of this cells do not require a prior recognition of surface structures of invading microorganism (Tort *et al*., 2003).

The humoral defense is composed by a variety of substances present on serum, mucus and eggs of fish that nonspecifically inhibit the growth of infections microorganisms. Lectins, transferrin, protease inhibitors, cytokines, lytic enzymes, such lysozyme, and a group of serum molecules that compose de complement system, are components of humoral defense.

Lysozyme is a bacteriolytic enzyme, widely distributed throughout the body, which acts against bacterial pathogens, eliminating them from the host. This protein is mainly produced by monocytes/macrophages and neutrophils and its response activity may vary according to the species and its location in the tissues (Uribe *et al*., 2011). The bactericidal action of this enzyme involves the hydrolyzation of the peptidoglycan of bacterial cell walls, causing cell lysis (Uribe *et al*., 2011). In case of gram-positive this enzyme acts directly killing the bacteria, but in gram-negative bacteria, this enzyme acts only after the complement and other enzymes have removed the outer cell wall, and consequently expose the inner layer peptidoglycans of bacteria (Iwama and Nakanishi, 1996). In addition to this direct effect on the bacteria, the lysozyme promotes phagocytosis and the action of complement system (Uribe *et al*., 2011). The complement is a part of humoral defense that plays an important role in fish defense against invading organisms such as fungi, viruses, parasites and bacteria. This system comprises a large

diversity of protein fragments present in the serum that when activated play a key role in the killing of microorganisms. One of the most important and best studied functions of the complement system is its ability to create pores in the cell walls of pathogens causing his death (Buchtíková *et al*., 2011; Holland and Sidings, 2002; Sunyer and Tort, 1995).

Given the ectothermic nature of fish, they more on their non-specific immune system, representing the most important mechanism in fish defense. In addition to this importance, the innate immune response has also a crucial role in the priming and regulation of the adaptive immunity (Lee *et al*., 2015).

Fish innate immunity starts with first barrier defenses such as mucus; it traps pathogens and includes lysozymes, antibacterial peptides which can eliminate pathogens.

Aims

Considering the rapid expansion and industrialization of aquaculture, there is a urgent need for new alternative ingredients to the traditional protein sources. However, for a correct evaluation, it is essential not only to study the effect of dietary inclusion of these new ingredients on growth and feed utilization but also on the general immune and health status of the fish. Besides, certain type of ingredients, such as yeast, may have certain immune modulating capacities, which may help to develop preventive methods against infectious outbreaks, reducing the use of chemotherapy in aquaculture productions systems. Sorghum dried distiller's grains with solubles (SDDGS) is an abundant by-product, produced locally at a very competitive price compared to soybean meal and corn. This ingredient has more protein and less fiber content than that of the corn DGGS, but also contain a high inclusion level of yeast. However, the effect of high replacement level of soybean meal by of this ingredient on general heath and immune status of tilapia was not yet investigated. Therefore, the present study was conducted to evaluate the effect of increasing levels of dietary SDDGS in of Nile tilapia (*Oreochromis niloticus*) diet on resistance, hematological and immune response to *Aeromonas hydrophila* challenge.

Material and methods

Experimental diets

Five diets were formulated to be isoproteic and isoenergetic, in digestible basis, attending to nutritional requirements of the species (NRC, 2011) (Table2).

Table 2 - Ingredients and proximate nutritional composition of the experimental diets.

	Levels of replacement (%) [1]				
	Control	**SDDGS10%**	**SDDGS30%**	**SDDGS50%**	**SDDGS70%**
Ingredients (*% dry matter*)					
Soybean meal	28.6	25.6	20.0	14.3	8.4
SDDGS	—	3.8	14.5	24.3	34.1
Poultry viscera meal	12.0	12.8	13.1	13.8	14.7
Corn gluten meal	10.0	10.0	10.0	10.0	10.0
Corn meal	42.3	40.7	34.3	28.8	23.2
Soybean oil	0.02	0.25	1.68	2.85	4.03
L-lysine	—	0.02	0.19	0.29	0.41
DL-methionine	0.13	0.13	0.12	0.09	0.06
L-threonine	0.30	0.29	0.30	0.31	0.33
L-tryptophan	0.04	0.08	0.09	0.12	0.15
Dicalcium phosphate	3.80	3.74	3.77	3.75	3.71
CMC[2]	2.00	1.72	1.03	0.42	-
Vitamin & mineral premix[3]	0.63	0.63	0.63	0.63	0.63
Vitamin C[4]	0.05	0.05	0.05	0.05	0.05
Antioxidant (BHT)[5]	0.02	0.02	0.02	0.02	0.02
Salt	0.10	0.10	0.10	0.10	0.10
Proximate composition (% dry matter)					
Dry matter (%)	96.8	95.1	94.8	94.0	94.0
Crude protein	29.6	30.3	31.4	30.9	31.5
Digestible protein*	26.8	26.8	26.8	26.8	26.8
Crude fat	4.6	5.6	5.7	8.6	9.8
Gross energy (kJ g^{-1} DM)	16.8	17.1	17.7	18.3	18.7
Digestible energy (kJ g^{-1})*	12.70	12.70	12.70	12.70	12.70
Crude fiber	3.63	3.71	3.59	3.71	3.86
Total calcium	1.84	1.95	1.83	2.03	1.87
Total phosphorus	1.01	1.12	1.04	1.00	1.08

[1]Replacement levels of protein soybean meal for the SDDGS;
SBM (crude protein: 73.6% DM; gross lipids: 11.5% DM).
[2]CMC = Carboxy methyl cellulose

Table 2 – To be continued

[3]Vitamin and mineral supplement (levels per kg of product): vitamin A = 1,200,000 IU; vitamin D3 = 200,000 IU; vitamin E = 12,000 mg; vitamin K3 = 2,400 mg; vitamin B1 = 4,800 mg; vitamin B2 = 4,800 mg; vitamin B6 = 4,000 mg; vitamin B12 = 4,800 mg; folic acid = 1,200 mg; calcium pantothenate= 12,000 mg; vitamin C = 48,000 mg; biotin = 48 mg; choline = 65,000 mg; nicotinic acid= 24,000 mg; Mn = 4.000 mg; Zn = 6.000 mg; I = 20 mg; Co = 2 mg; Cu = 4 mg e Se = 20 mg;
[4]Vitamin C Rovimix@ Stay-35, DMS Nutritional Products, Switzerland;
[5]Butylated hydroxytoluene;
*Determined based on the digestibly coefficients of each ingredient used (according to Furuya *et al*., 2010)

A control diet was formulated without the incorporation of SDDGS. In test diets, soybean meal and corn meal were replaced by SDDGS at the level of (10, 30, 50 and 70% SDDGS) on an equal digestible protein basis. Digestible protein coefficients of soybean meal and SDDGS were previously estimated as 94.4% and 66.4%, respectively. Carboxy methyl cellulose was use to equalized the fiber content of all the dietary treatments

For manufacturing each of the experimental diets, all dietary ingredients were finely ground, well mixed with water (± 60.0 °C) in a proportion of 20 % of dry weight. Then the mixture was extruded at (90° C) on a single-screw laboratory extruder and dried in a fan oven (± 55.0 °C) for 24 hours. Diets were stored at 4 ºC until further use. During extrusion, the diameter of pellets were calibrated according to the size of the fish under study.

Growth Trial

The growth trial was performed at the nutrition and fish health laboratory - Aquanutri, UNESP, Botucatu, Brazil. Nile tilapia juveniles were obtained from local commercial farm and acclimated to the experimental facilities for 2 weeks before (Figure 6). Then, 220 healthy Nile tilapia juveniles (32.8 ± 1.0g) were randomly distributed in twenty fiberglass tanks of 250L water capacity (eleven fish per tank) (Figure 7). Tanks were supplied with flow-through dechlorinated, heated freshwater at an initial rate of about 3L/min and increased gradually to about 5 L/min prior to the end of the study. Water was continuously aerated using air stones. Water temperature, dissolved oxygen and ammonia level were checked weekly. During the trial, water temperature was maintained at 26.5 ± 1.3°C, the photoperiod at 12:12h light:dark schedule and the dissolved oxygen averaged 6.5 g/l.

Figure 6 – Acclimatization of Nile tilapia juveniles to experimental facilities. Photography by Pedro Pucci.

Fish were fed the five experimental diets in quadruplicate, by hand, two time a day, to apparent satiation, 7 days a week during 90 days. At the end of the growth trial, pre-challenge blood and serum samples were collected from twenty fish, four per diet.

Growth performance and feed efficiency data were not the scope of the present study and are not presented.

Figure 7 - Growth system. Photography by Rafael de Almeida

Bacterial growth and inoculum preparation

After all the sampling work of the growth trial, the remaining fish were used in a bacterial challenge. Before it, a bacteria suspension was prepared. For that, *Aeromonas hydrophila*¸ from a virulent outbreak of hemorrhagic septicemia in Nile tilapia (Jaboticabal, SP, Brazil), was grown in Brain-heart infusion (BHI, Bacto™) at 18°C for 24h. The optimum *A. hydrophila* concentration to be use in the challenge trial was adjusted, using McFarland scale, to a concentration of 1.8×10^5 colony-forming units (CFU) ml^{-1}.

Bacterial Challenge

The bacterial challenge was performed in another independent system, consisted of twenty five plastic tanks of 40L water capacity (Figure 8), placed in a proper isolated room, connected to an independent filtered, aerated and supplied with a continuous flow of water (1.2 L/min) (Figure 9). Heaters were placed into each tanks and connected to a central heating system, to maintenance temperature during the experimental period.

Figure 8 - *Challenge room. Photography by Rafael de Almeida*

Figure 9 - Filter system. Photography by Rafael de Almeida.

At the conclusion of the growth trial, 50 fish, 10 per diet per diet (average individual weight of 160.3 ± 26.7g) were anaesthetized with benzocaine (100 mg L^{-1}) and challenged by intraperitoneal injection with 100 µl of *A. hydrophila* solution containing 1.0 x 10^6 CFU ml^{-1} (predetermined LD_{50} concentration; concentration lethal to 50% of exposed fish). The desired bacterial concentration was prepared in sterile medium by 1:10 serial dilution. After injection, 25 groups of 2 fish were randomly allocated in each experimental tank. Twenty-four hours after injection, each group of fish was fed by hand, twice daily with the same experimental diet assigned in the growth trial. Fish mortality was recorded twice a day for 15 days and all dead fish were photographed. At the end

of the challenge period, from the surviving fishes, post-challenge blood and serum samples were collected for all hematological and immunological parameters, following the same protocol as previously indicated. During the challenge period, water temperature was maintained at 25 ± 0.5 °C and photoperiod maintained at 8:16h light:dark schedule.

Hematological procedures

Blood samples were collected from 6 fish per treatment, before challenge, and from 5 to live fish after challenge (depending on the mortality rate for each diet). Fish were kept unfed for one day. Then, fish were anesthetized with benzocaine (100 mg L^{-1}) and blood was collected from caudal vein using a tuberculin syringe, rinsed with anti-coagulant (3% EDTA) (Figure 10). The hematological profile consisted of total red (RBC) and white (WBC) blood cells counts, hematocrit (Ht) and hemoglobin (Hb). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were also calculated as follows:

- MCV (fL) = (Ht/RBC) x 10
- MCH (pg $cell^{-1}$) = (Hb/RBC) x 10
- MCHC (g dL^{-1}) = (Hb/Ht) x 100

Figure 10 - Blood collection from caudal vein. Photography by Rafael de Almeida.

Hemoglobin (Hb) was determined by the cyanomethemoglobin colorimetric method using a commercial kit (Labtest Diagnostic®, Lagoa Santa, MG, Brazil) according to (Weiss and Wardrop, 2011). The hematocrit (Ht) percentage was determined using the microhematocrit method described by (Goldenfarb *et al*., 1971).

Preparation and examination of stained blood smears

To perform the differential counting of leucocytes, immediately after blood collection, blood smears were performed from homogenized blood, air dried, and stained with May-Grünwald-Giemsa. The slides were examined (1000x) and two hundred cells were counted to establish the percentage of leucocytes and thrombocytes. Thereafter 200 leucocytes were counted and classified as lymphocytes, monocytes and neutrophils (Figure 11). The relative percentage and absolute value ($x10^4\ \mu l^{-1}$) of each cell type was calculated according to (Ranzani-Paiva etl al., 2013).

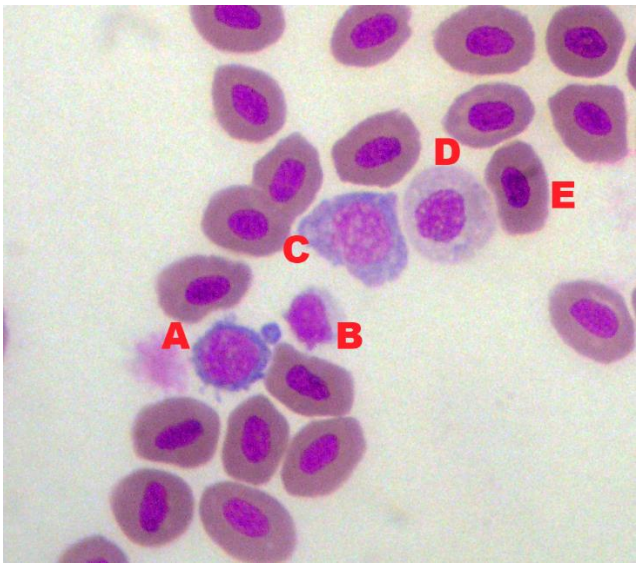


Figure 11 - Blood smear stained with May-Grünwald-Giemsa. Letters are: (A) Lymphocyte; (B) thrombocyte; (C) monocyte; (D) neutrophil; (E) Erythrocytes. Optic microscope photography (1000x) by Rafael de Almeida.

The remaining blood was centrifuged at 3000 rpm during 10 min and plasma stored at 20 ◦C until analysis for plasma protein, albumin and humoral parameters measurements

Plasma protein and albumin analytical procedures

Total plasma protein (Tpp) was measured using a manual Goldberg refractometer by breaking the microhematocrit capillary just above the leucocyte layer after the hematocrit reading (Weiss and Wardrop, 2011). The albumin concentration (ALB) was determined by the bromocresol method using the commercial kit Albumina Labtest Diagnostic® for colorimetric determination. The albumin/globulin ratio (A/G) was

determined using ALB and TPP values (A/G=ALB/Globulin; Globulin=TPP-ALB;). Glucose analyses were performed using Glucose Liquiform Labtest Diagnostic® kit.

Humoral parameters analytical procedures

Lysozyme activity

Lysozyme activity was estimated using a turbidimetric assay based on the method described Ellis *et al*. 1990. Summarily, a solution of *Micrococcus lysodeikticus* (0.5 mg mL^{-1}, 0.05 M sodium phosphate buffer; pH 6.2) was prepared. To a microplate, 15 µl of plasma and 250 µl of the above suspension were added to give a final volume of 265 µl. The reaction was carried out at 25 °C and the absorbance (450 nm) was measured after 0.5 and 4.5 min in a microplate reader (Synergy HT; Biotek). Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve. All analyses were conducted by triplicates.

Alternative complement pathway

Alternative complement pathway activity was estimated as described by Sunyer and Tort (1995). The buffers used in this method were the following: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, similar to the previous one but adding 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mgþ2 and 10 mM EGTA. To perform the ACP determination, rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.8 x 108 cells ml-1.Ten µl of RaRBC suspension were then added to 40 µl of serially diluted plasma in Mg-EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with regular shaking. In order to stop the reaction it was added 150 µl of cold EDTA- GVB. Samples were then centrifuged (1000 rpm) during 2.5 min and the extent of hemolysis was estimated in a microplate reader (Synergy HT, Biotek) by measuring the optical density of the supernatant at 414 nm. The alternative complement pathway activity (ACH50) units were described as the concentration of plasma giving 50% hemolysis of RaRBC. Triplicates were used to conduct all analyses.

Data analysis

To compare control diet versus each test diet, after the bacterial challenge, fold change levels (means ± SD) were calculated for all parameters by dividing each parameter value by the mean value from control treatment minus one. Fold values higher than 0 express an increase and lower than 0 a decrease in the parameters assessed relative to fish from control treatment.

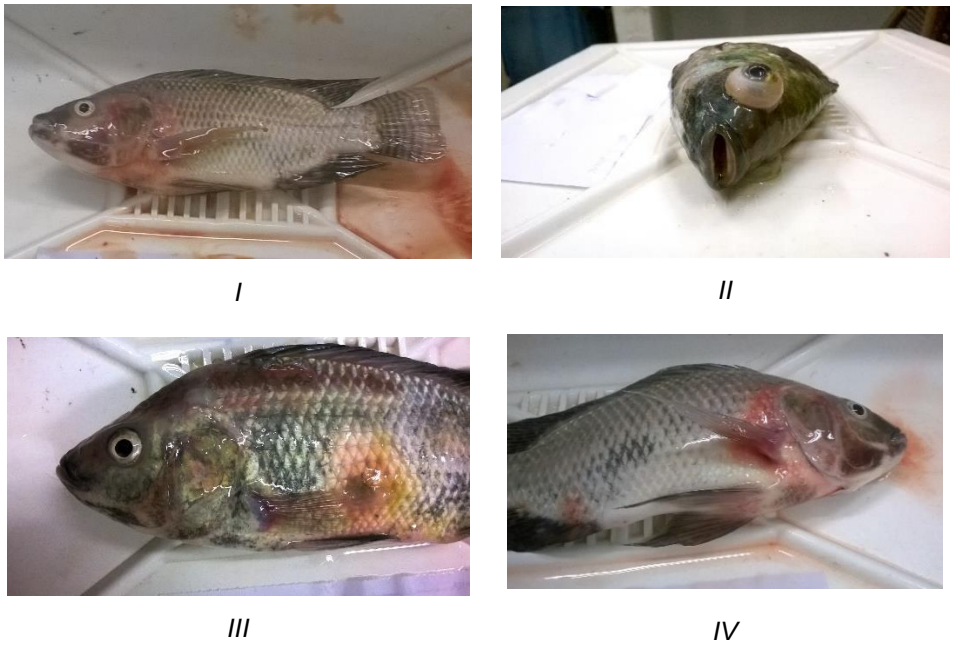
Statistical Analysis

All results are expressed as means ± standard deviation (SD). Data were analysed for normality and homogeneity of variance by Levene test and, when necessary, they were transformed before being treated statistically. To compare the effects of various dietary treatments on all the parameters tested, data were subjected to one-way analysis of variance (ANOVA). All statistical analyses were performed using the computer package SPSS 23 for WINDOWS.

Results

Bacterial Challenge

During the bacterial challenge, different abnormal fish behaviors were recorded, such as erratic swimming, loss of appetite, lethargy or external alarm status. On the other hand, some fish did not show any symptom of infectious disease. Moreover, all the fish that died presented symptoms associated to an infection by *Aeromonas hydrophila* such as hemorrhagic septicemia (Figure 12-I), exophthalmia (Figure 12-II), skin ulcerations (Figure 12-III) and abdominal bloating (Figure 12-IV).



I *II*

III *IV*

Figure 12 - General symptoms of fish that died during bacterial challenge with *Aeromonas hydrophila.* Photography by Rafael de Almeida

In terms of the number of days to first mortality, after the *Aeromonas hydrophila* challenge, fish fed the control and SDDGS 30% diets died earlier, after 1.2 days, followed by fish fed the SDDGS 10% and SDDGS 70% diets, after 2.8 and 5.4 days, respectively. However, fish fed the SDDGS 50% diet registered the first mortality only 6.7 days after challenge. The survival rate, by day 15 after challenge, was higher for SGGDS 30% and SDDGS 50% diets, averaging 70%, than that of the other diets, which averaged 50-60% (Table 3).

Table 3 - Mean and SD of number of days to first mortality and survival rate of Nile Tilapia 15d post-challenge with *Aeromonas hydrophila* I.

Diet	Days to first mortality	Survival (%)
Control	1.2 ± 0.4 [a]	50 [a]
SDDGS 10%	2.8 ± 3.5 [a]	60 [a]
SDDGS 30%	1.2 ± 0.5 [a]	70 [a]
SDDGS 50%	6.7 ± 0.5 [a]	70 [a]
SDDGS 70%	5.4 ± 4.3 [a]	50 [a]

[I] Values are means of two fish per tank and five tanks per treatment. No significant differences were observed among treatment means ($P>0.05$).

A quadratic relationship between dietary SDDGS incorporation level and survival after bacterial challenge was observed (Figure 13). Maximum survival rate of 71.9% would be achieved by the dietary inclusion level of 35.5% of SDDGS.

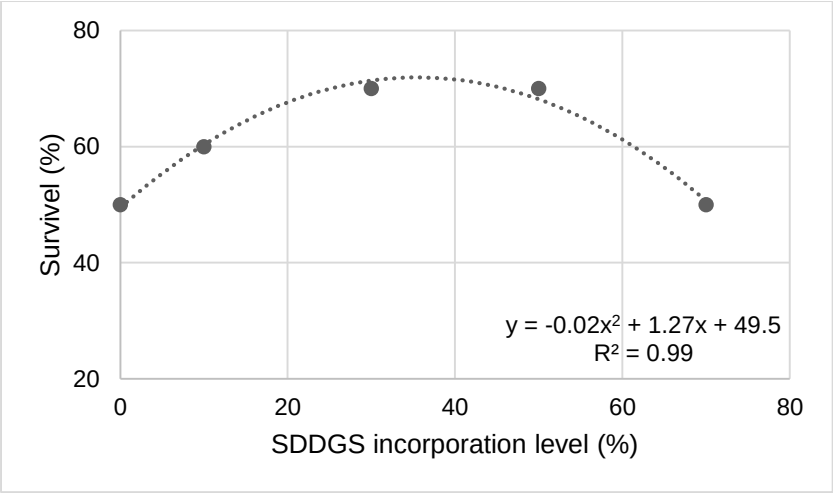


Figure 13 - Relationship between SDDGS incorporation level (%) and the survival rate (%)

Hematological and immune parameters

Hematological parameters of fish fed the experimental diets, before and after bacterial challenge, are presented in table 4. No statistical differences among the experimental treatments were observed as well as comparing each parameter, before and after challenge.

Table 4 - Select hematologic analytes of Nile tilapia fed experimental diets, before and after challenge with *Aeromonas hydrophila*.

Parameters		**Dietary treatments**				
		Control	**SDDGS10%**	**SDDGS30%**	**SDDGS50%**	**SDDGS70%**
RBC (106 µl-1)	**Before**	1.66 aA ±0.18	1.18 aA ±0.18	1.70 aA ±0.16	1.75 aA ±0.18	1.7 aA ±0.17
	After	1.62 aA ±0.88	1.56 aA ±0.27	1.48 aA ±0.21	1.36 aA ±0.15	1.62 aA ±0.14
Hb (g dl-1)	**Before**	6.81aA ±0.84	7.18 aA ±0.74	7.04 aA ±0.64	6.75 aA ±0.48	6.71 aA ±0.62
	After	6.16 aA ±0.27	5.51 aA ±0.52	5.81 aA ±0.69	5.56 aA ±0.72	6.33 aA ±0.79
Ht (%)	**Before**	26.17 aA ±2.88	29.06 aA ±2.82	29.06 aA ±2.09	26.62 aA ±1.99	26.87 aA ±2.69
	After	29.20 aA ±2.41	26.75 aA ±2.22	26.60 aA ±2.90	25.54 aA ±3.36	28.87 aA ±2.04
MCV (µm3)	**Before**	157.70 aA ±10.95	163.83 aA ±9.70	171.93 aA ±19.90	156.70 aA ±19.86	153.88 aA ±17.40
	After	179.35 aA ±8.70	174.07 aA ±20.79	180.91 aA ±13.79	182.87 aA ±10.38	183.50 aA ±10.77
MCH (pg cell-1)	**Before**	40.97 aA ±2.79	40.22 aA ±2.60	41.49 aA ±3.18	39.70 aA ±4.27	38.46 aA ±4.44
	After	37.93 aA ±1.93	35.99 aA ±4.31	39.55 aA ±4.10	40.92 aA ±3.95	38.86 aA ±3.64
MCHC (g 100ml-1)	**Before**	26.00 aA ±1.04	24.73 aA ±1.55	24.27 aA ±2.03	25.43 aA ±1.72	25.03 aA ±1.68
	After	21.19 aA ±1.55	20.68 aA ±1.74	21.83 aA ±1.01	21.73 aA ±1.10	21.28 aA ±1.58
WBC (104 µl-1)	**Before**	2.85 aA ±0.70	2.84 aA ±0.81	4.37 aA ±1.94	3.67 aA ±2.34	2.44 aA ±1.24
	After	2.76 aA ±1.40	2.05 aA ±0.93	2.20 aA ±0.65	2.75 aA ±2.66	2.99 aA ±1.68
Lym (104 cells µL-1)	**Before**	2.50 aA ±0.67	2.49 aA ±0.84	3.78 aA ±1.25	3.46 aA ±2.34	2.12 aA ±1.24
	After	2.29 aA ±1.29	1.83 aA ±0.96	1.75 aA ±0.74	2.28 aA ±2.24	2.58 aA ±1.52
Mon (104 cells µL-1)	**Before**	0.11 aA ±0.08	0.13 aA ±0.12	0.24 aA ±0.31	0.10 aA ±0.04	0.11 aA ±0.04
	After	0.24 aA ±0.09	0.12 aA ±0.07	0.17 aA ±0.12	0.24 aA ±0.27	0.21 aA ±0.15
Neut (104 cells µL-1)	**Before**	0.23 aA ±0.23	0.21 aA ±0.17	0.35 aA ±0.51	0.10 aA ±0.03	0.20 aA ±0.06
	After	0.21 aA ±0.13	0.09 aA ±0.08	0.27 aA ±0.25	0.22 aA ±0.19	0.18 aA ±0.18
Trb (104 cells µL-1)	**Before**	2.96 aA ±1.71	3.43 aA ±1.95	1.84 aA ±1.05	3.68 aA ±2.07	5.24 aA ±0.96
	After	1.24 aA ±1.14	2.28 aA ±1.23	2.66 aA ±2.09	1.76 aA ±0.94	2.27 aA ±1.46

[I] Means± standard deviations. Small letter: comparison among treatments with moments fixed; capital letter: comparison between moments with treatments fixed.
Ery: erythrocytes; Hb: hemoglobin; Htc: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; Leuc: leucocytes; Lym: lymphocytes; Mon: monocytes; Neut: neutrophils; Trb: thrombocytes.

Total number of erythrocytes ranged between 1.18 and 1.75x10^{6}µl^{-1}. Before bacterial challenge, mean levels of Hb, Ht and MCV were higher for SDDGS 10% and SDDGS 30% compared to the other treatments, however not statistically significant. Total number of leukocytes ranged from 2.44 to 4.37x10^{4}µl^{-1}, being highest for SDDGS 30% diet. Total thrombocytes number ranged between 1.84 and 5.24x10^{4}µl^{-1}, corresponding to the SDDGS 30% and SDDGS 70% diet, respectively. Fish fed the SDDGS 30% diet registered the higher differential count of leucocytes (lymphocytes: 3.78x10^{4} µL^{-1}; monocyte: 0.24 x10^{4} µL^{-1} and neutrophils: 0.35 x10^{4} µL^{-1}), comparatively to other dietary treatments.

After bacterial challenge, mean levels of Ht, Hb, MCV, MCH and MCHC were similar between all treatments. Total number of erythrocytes ranged between 1.36 and 1.62x10^{6}µl^{-1} and the leukocyte total number ranged from 2.05 and 2.99 x10^{4}µl^{-1} having been reached the maximum number of cells with the SDDGS 70% diet. Total thrombocytes ranged between 1.2 and 2.7x10^{4}µl^{-1}, with the control and SDDGS 30% diets, respectively. In terms of differential count of leucocytes, diet SDDGS 30% registered the highest number of neutrophils (0.35 x10^{4} µL^{-1}) and the lowest number of lymphocytes (1.75 x10^{4} µL^{-1}) comparatively to other treatments.

A quadratic relationship between total number of leucocytes, before bacterial challenge, and the dietary SDDGS incorporation level was observed (Figure 14). According to it a maximum leucocyte concentration (4.07 x 10^{4} µl^{-1}) would be attained with the incorporation level of 33.4% of SDDGS.

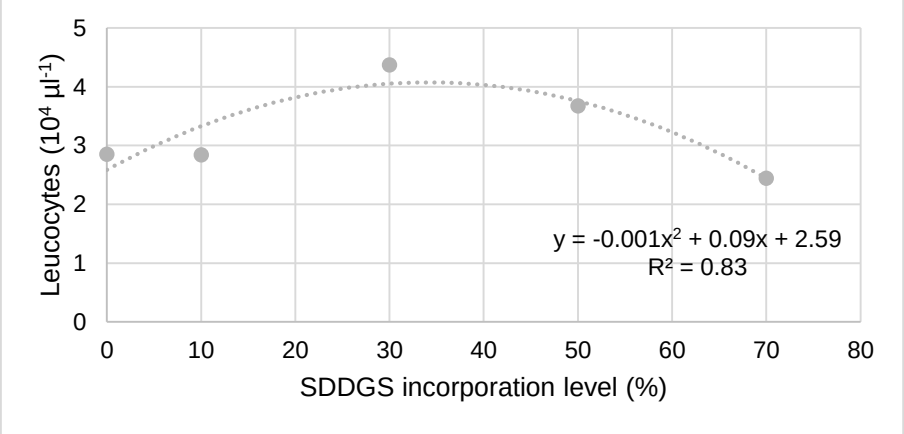


Figure 14 – Relationship between the SDDGS incorporation level (%) and the leucocytes concentration (10^{4} µl^{-1}) before bacterial challenge.

Plasma metabolites profile and innate immune parameters of fish fed the experimental diets, before and after the bacterial challenge, are presented in Table 5. No statistical differences among the experimental treatments were observed as well as comparing each parameter, before and after challenge.

Table 5 - Serum chemistry analyses of Nile tilapia fed experimental diets, before and after challenge with Aeromonas hydrophila

Parameters		**Dietary treatments**				
		Control	**SDDGS10%**	**SDDGS30%**	**SDDGS50%**	**SDDGS70%**
TPP (mg dL-1)	**Before**	2.85 aA ±0.53	3.05 aA ±0.46	2.80 aA ±0.38	2.81 aA ±0.33	2.85 aA ±0.39
	After	3.23 aA ±0.28	2.92 aA ±0.21	2.87 aA ±0.48	3.51 aA ±0.99	3.35 aA ±0.47
Glucose (mg dL-1)	**Before**	54.06 aA ±19.47	57.25 aA ±12.58	61.03 aA ±11.62	69.36 aA ±16.80	55.07 aA ±13.76
	After	63.19 aA ±7.87	62.60 aA ±7.44	68.20 aA ±20.48	64.30 aA ±12.95	55.24 aA ±33.80
Albumin (mg dL-1)	**Before**	0.98 aA ±0.24	0.97 aA ±0.18	0.97 aA ±0.18	1.03 aA ±0.14	1.14 aA ±0.25
	After	0.87 aA ±0.24	0.81 aA ±0.06	1.08 aA ±0.26	1.06 aA ±0.39	1.18 aA ±0.27
Globulin (mg dL-1)	**Before**	1.86 aA ±0.34	2.08 aA ±0.52	1.95 aA ±0.49	1.77 aA ±0.31	1.87 aA ±0.42
	After	2.28 aA ±0.25	2.10 aA ±0.21	1.79 aA ±0.50	2.11 aA ±0.43	2.21 aA ±0.20
A:G (mg dL-1)	**Before**	0.53 aA ±0.10	0.49 aA ±0.18	0.48 aA ±0.22	0.60 aA ±0.15	0.71 aA ±0.27
	After	0.39 aA ±0.12	0.39 aA ±0.05	0.66 aA ±0.32	0.51 aA ±0.12	0.50 aA ±0.09
ACH50 (units ml-1)	**Before**	166.64 aA ±61.64	191.57 aA ±53.74	213.71 aA ±30.33	165.18 aA ±41.51	224.14 aA ±21.52
	After	201.35 aA ±65.45	215.82 aA ±3.63	166.25 aA ±18.51	194.83 aA ±38.59	165.17 aA ±9.98
Lysosyme (µg ml-1)	**Before**	6.35 aA ±2.96	7.66 aA ±3.04	4.50 aA ±2.05	5.87 aA ±2.35	3.57 aA ±2.02
	After	5.46 aA ±1.89	6.47 aA ±2.16	7.05 aA ±1.18	3.86 aA ±4.23	4.35 aA ±0.92

[I] Means± standard deviations. Small letter: comparison among treatments with moments fixed; capital letter: comparison between moments with treatments fixed.
TPP: plasmatic protein; Glu: glucose; Alb: albumin; Glob: globulin; A/G: albumin/globulin ratio; ACH50: alternative complement activity; Lyz: lysozyme.

Before bacterial challenge, total plasma protein, albumin, globulin concentrations and A:G ratio did not differed among the dietary treatments. Minimum plasma glucose level was obtained with the control diet and the maximum with the SDDGS 50% diet. Plasma alternative complement activity ranged from 165.18 to 224.14 units ml-1, for diet SDDGS 50% and SDDGS 70%, respectively. Lysozyme activity ranged from 3.57 and 7.66 µg ml-1, for diet SDDGS 70% and SDDGS10%, respectively, reveling a tendency to decrease with the increase of dietary inclusion of SDDGS.

After bacterial challenge, total plasma protein, albumin, globulin concentrations and A:G ratio did not differed among the dietary treatments. Glucose concentration mean values varied between 55.24 and 68.20 mg dL^{-1}, for diets SDDGS 70% and SDDGS 30%, respectively. Plasma alternative complement activity ranged from 165.17 to 215.82 units ml^{-1}, for diets SDDGS 70% and SDDGS 10%, respectively. Lysozyme activity ranged from 3.86 and 7.05 µg ml^{-1} for diets SDDGS 50% and SDDGS 30%, respectively. After challenge lysozyme activity tend to increase with the dietary increased of SDDGS up to 30%, but it drastically decreased for high inclusion levels.

After challenge, the fold changes of hematological and humoral parameters values of control diet versus each experimental diet were determined and are presented in Figure 15, 16, 17, 18, 19 and 20. No significant differences were identify between dietary treatments and control diet. Even though the total RBC trend to decreed with the dietary inclusion of SDDGS (Figure 16), the MCV, MCH and MCHC trended to increase with 30 and 50% of SDDGS inclusion (Figure 15). Moreover, total thrombocytes number also increased with the SDDGS inclusion relatively to the control diet (Figure 16); for diet 30% SDDGS diet it more than double the total number (fold change value = 1.10). Relatively to the control diet, the incorporation of 30% SDDGS increase the total number of neutrophils (fold change value = 0.5; Figure 17). Also the plasma total protein, albumin and A:G ratio were increased with the incorporation of 30% of SDDGS, but globulin was decreased (Figure 18).

Considering the humoral parameters, fold change values indicate an increase of lysozyme concentration with the inclusion of 10% and 30% of the SGGDS relative to control diet (Figure 19) and a decreased of the alternative complement activity by the dietary inclusion of 30 to 70% SDDGS in the control diet (Figure 20).

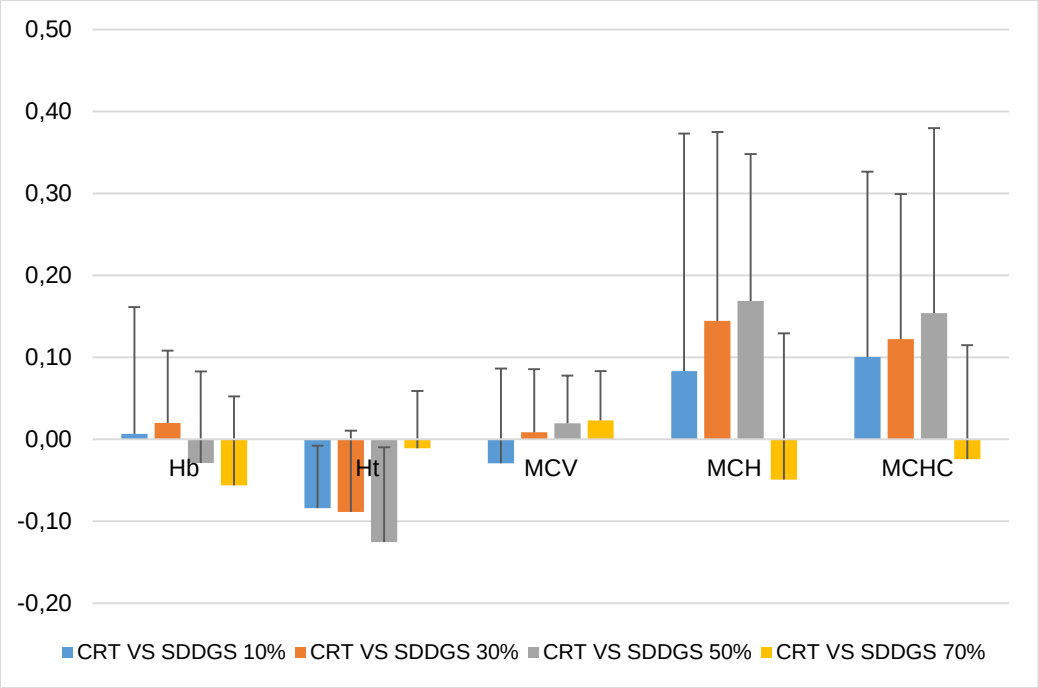


Figure 15 - Fold change values of hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) of Nile tilapia fed each test diet relatively to the control diet,15 day after challenge. Values (means ± SD) were calculated by dividing each parameter value by the mean value from control treatment minus one. No significant differences were observed among treatment means (*P*>0.05).
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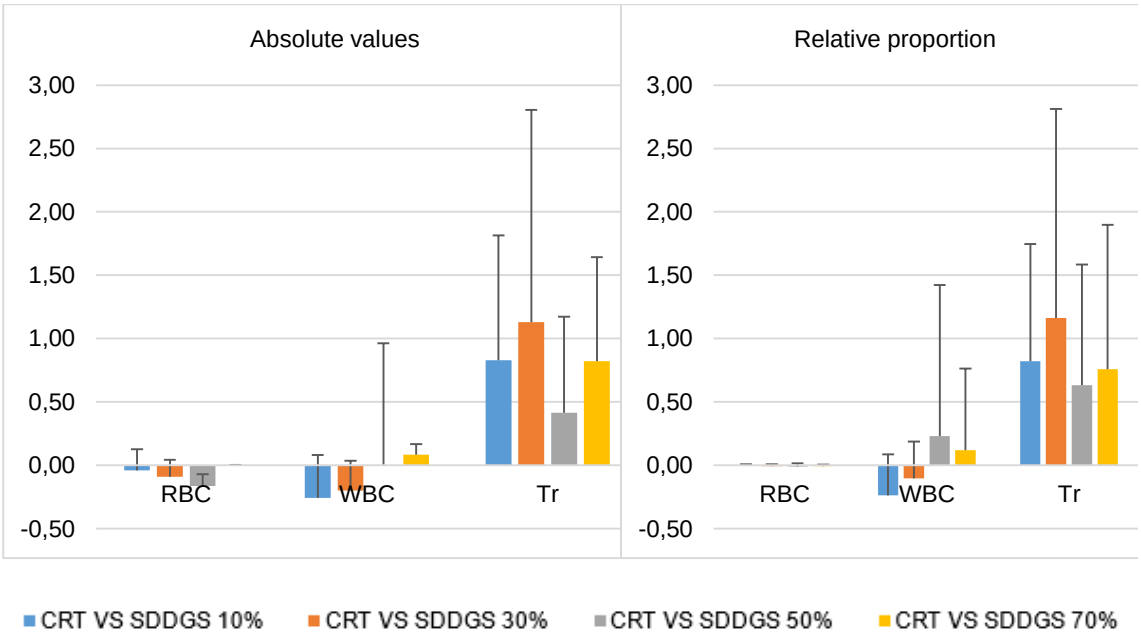

Figure 16 - Fold change values of relative proportion and absolute values of red blood cells (RBC), white blood cells (WBC) and Thrombocytes (TR) relative proportion and absolute values of Nile Tilapia fed each test diet relatively to the control diet,15 day after challenge. No significant differences were observed among treatment means (*P*>0.05).

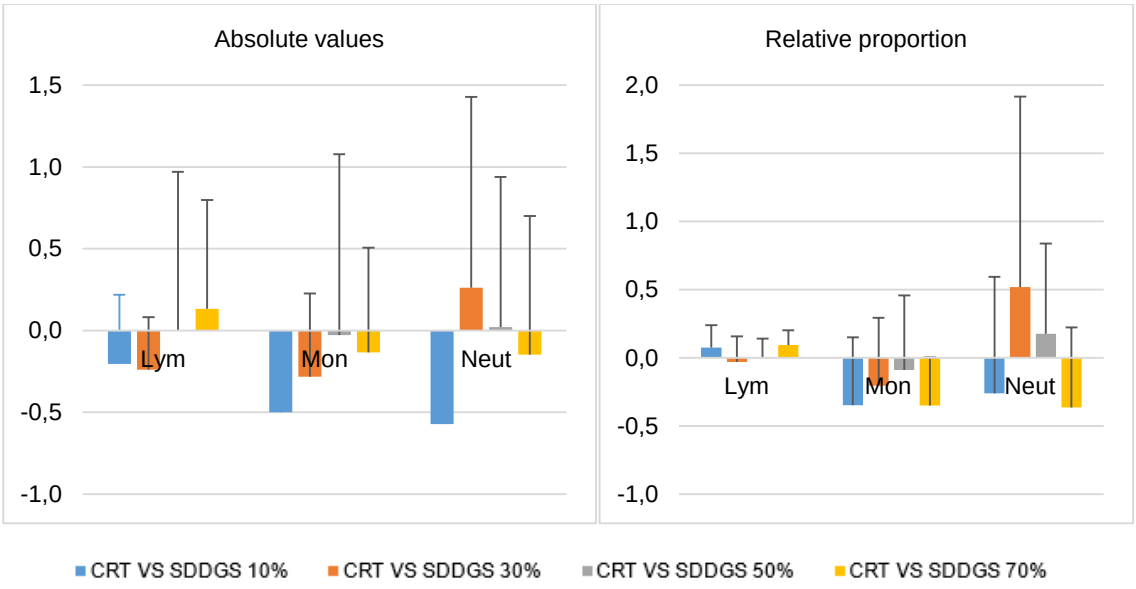

Figure 17 - Fold change values of relative proportion and absolute values of blood leucocytes (lymphocytes (Lym). monocytes (Mon) and neutrophils (Neut) in Nile tilapia fed each test diet relatively to the control diet,15 day after challenge. Values are expressed as means ± SD (n = 5). No significant differences were observed among treatment means (*P*>0.05).

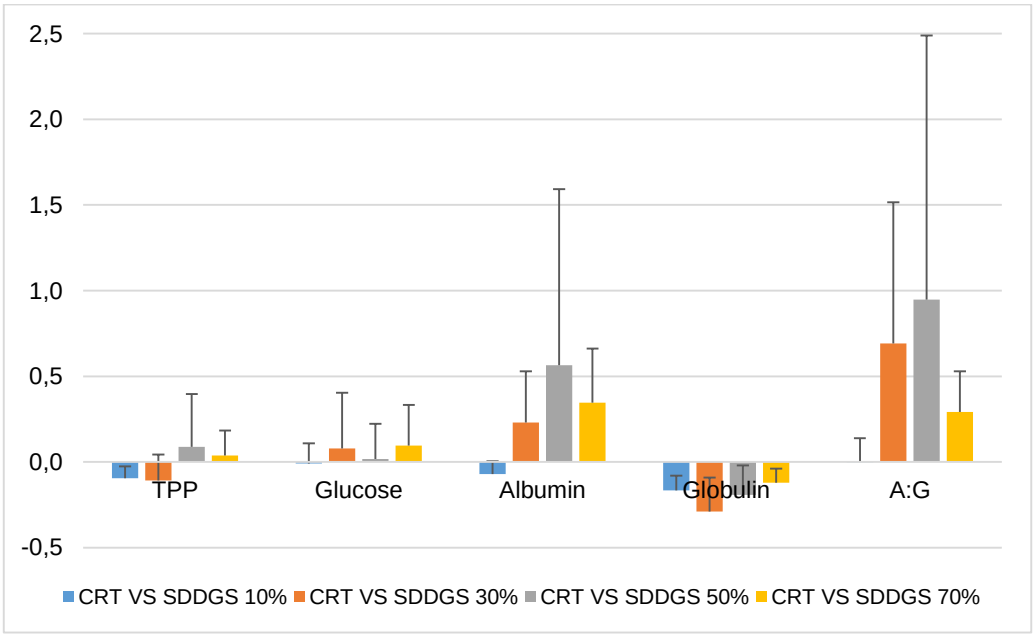

Figure 18 - Fold change values of total plasma protein, albumin, glucose and globulin levels and albumina:globulin ratio (A:G) in Nile tilapia fed each test diet relatively to the control diet,15 day after challenge. Values are expressed as means ± SD (n = 5). No significant differences were observed among treatment means (*P*>0.05).

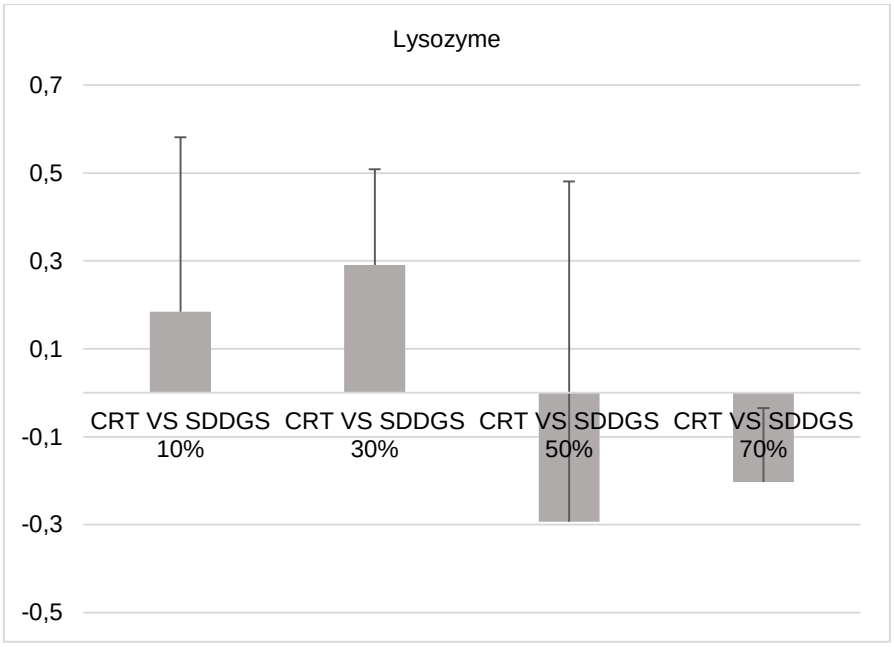

Figure 19 - Fold change values lysozyme concentration in Nile tilapia fed each test diet relatively to the control diet, 15 day after challenge. Values are expressed as means ± SD (n = 3). No significant differences were observed among treatment means (*P*>0.05).

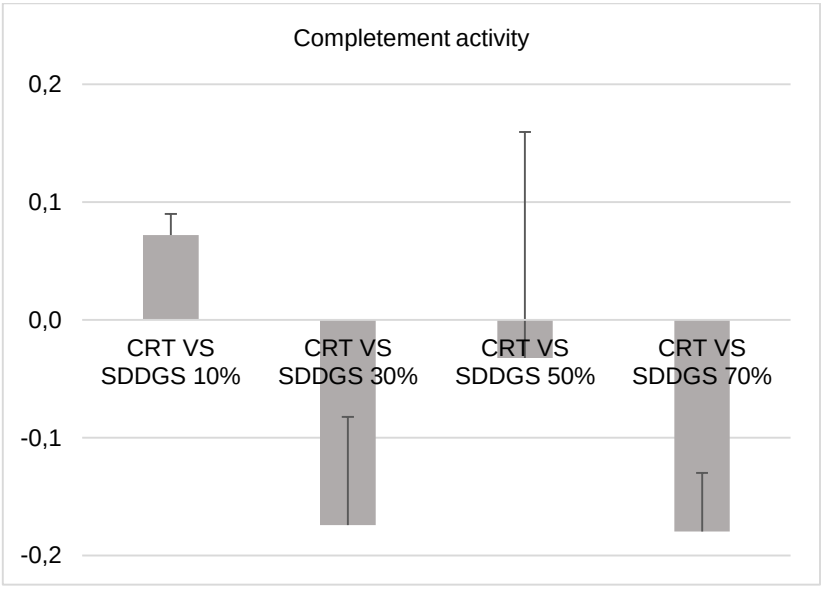

Figure 20 - Fold change values of alternative complement activity (ACH50) in Nile tilapia fed each test diet relatively to the control diet,15 day after challenge. Values are expressed as means ± SD (n = 3). No significant differences were observed among treatment means (*P*>0.05).

Discussion

One of the major challenge of tilapia production is the development of commercial, cost effective tilapia feeds using locally available, cheap and unconventional feed resources z. Under these situations, alternative protein sources like distillers dried grain with solubles (DDGS) may be important. Distillers dried grain with solubles are a by-product of ethanol industry, which are not used for human consumption, is cheaper than other traditional ingredients, such as soybean meal, and is widely available in many parts of the world.

Compared to the most common DDGS, the corn DDGS, sorghum DDGS is slightly higher in crude protein, lower in crude fiber and lipid compared to corn DDGS (Welker *et al*., 2014a). With regard to amino acid composition, levels of methionine and threonine are similar, lysine and arginine are lower and tryptophan levels are substantially higher in sorghum DDGS compared do corn DDGS (U.s.Grains Council, 2012). The nutritional profile of both DDGS, corn and sorghum, may lead to differences in optimum incorporation levels in diets as well as different repercussion on growth performance, feed utilization and well-being of fish.

For tilapia it has been demonstrated that DDGS may be incorporated in diets up to 20-30% without compromising its growth performance and feed utilization (Wu *et al*., 1996, 1997; Lim *et al*., 2006; Schaeffer *et al*., 2010). However, to date, DDGS effects on general well-being and health status have been inconsistent (Welker *et al*., 2014b). Even though, some characteristics of DDGS, like its high fiber content, phytic acid, and low to moderate digestibility (U.S.GrainsCouncil., 2012) may impaired welfare and health of fish. Thus it is necessary to gain deeper insight on the possible modulation action of the dietary incorporation of DDGS on the immune status, stress and disease resistance. Moreover, due to the presence of the yeast used in DDGS ethanol production process, as well as the presence of on-starch polysaccharides fraction, DDGS may have probiotic/prebiotic effect that deserves to be explored (Schaeffer *et al.,* 2010). Indeed, DDGS contain yeast cells and, in general, yeast protein represents about 5.3% of the total protein content of DDGS (Lim *et al*., 2011). Yeast is a nutritious ingredient containing protein, vitamins of B-complex, and many biologically active compounds with potential immunological effect, including β-glucans, mannan-oligosaccharides, chitin, nucleotides, and glutamate (Lara-Flores *et al.,* 2003; Oliva-Teles, 2012; Navarrete and Tovar-Ramirez, 2014, Lee *et al*., 2015).

Recent studies indicate that diets containing small quantities of yeast enhance resistance to bacterial infections, decreasing mortality, compared with basal diets (Li and Gatlin., 2004; Reyes-Becerril *et al*., 2008). A study conducted by Abdel-Tawwab *et al*. (2008) carried out to evaluate the use of commercial live bakers' yeast *Saccharomyces cerevisiae* in diets fed Nile tilapia indicated that the addition of different percentages of baker's yeast to diets of Nile tilapia (averaged body weight of 0.3 g) significantly reduced mortality (40–60%) after intraperitoneal injection of *A. hydrophila* compared to fish fed a diet without baker's yeast.

In a study conducted by He *et al*. (2009), the addiction of yeast fermentation products to diets fed to hybrid Tilapia lead to an increase of serum lysozyme activity, head kidney macrophage phagocytic activities, and macrophage respiratory burst activity compared to fish fed a diet without the yeast product. However, this study did not test the response to a bacterial challenge and so it is unclear if the increased levels of non-specific immunity would have biological significance (Lee *et al*., 2015).

β-glucans have been reported to stimulate the non-specific immune responses, such macrophage and neutrophil migration and phagocytosis, while yeast glucan has been shown to enhance lysozyme activity (Santarém *et al*., 1997; Refstie *et al*., 2010; Selim and Reda, 2015). Moreover, prolonged feeding of β-glucans has been reported to decrease the susceptibility to bacterial infection (Barros *et al*., 2014; Lee *et al*., 2015). In Nile tilapia, Barros *et al*. (2014) observed that dietary supplementation with β-glucans improved fish resistance against stressful factors and Aeromonas *hydrophila* challenge.

Specific mechanism by which yeast and yeast supplements (β-glucans) influence immune function are currently unknown, even though it is believed that the microflora of the intestine is determinant for the development of the immune system in fish, possibly due to increased levels of immunoglobulin in fish fed a diet containing yeast (Lee *et al*., 2015)

For terrestrial animals, it has been observed that a moderate inclusion of DDGS may improve the immune function. In growing pigs, it seems that dietary inclusion of 10% of DDGS may reduce the severity of intestinal lesions due to bacterial challenge (Whitney *et al*., 2006; Perez, 2010), as well as increased expression of both pro-inflammatory and anti-inflammatory cytokines in intestinal tissue (Weber *et al*., 2008). For broilers fed up to 15% of DDGS, it has been observed an improvement of hematological and histological parameters (Youssef *et al*., 2013).

Lim *et al.* (2009) studied the partial replacement of soybean and corn meal by DDGS in diets for channel catfish and observed that diets based on DDGS, regardless the inclusion ratio, increased the hemoglobin and hematocrit levels. Also it was observed that the dietary inclusion of 20-40% of DDGS increased serum total immunoglobulin (Lim *et al.,* 2009). However, for tilapia the same authors obtained contradictory results. Indeed, in a similar study performed with Nile tilapia it was observed that dietary DDGS inclusion did not significantly affect the level of hemoglobin, hematocrit, red blood cell count, white blood cell count, serum protein, lysozyme active and antibody titer among treatments (Lim *et al.,* 2007). Likewise, Shelby *et al*. (2008) studied the effect of DDGS on immune function and disease resistance of Nile tilapia to *Streptococcus iniae* challenge and reported that there were no significant differences in percentage survival in fish after challenge with *Streptococcus iniae* among treatments, as well as in the number of erythrocytes and leucocytes, respiratory burst activity, total plasma protein, globulin, lysozyme, alternative complement activity. Schaeffer *et al*., (2012) studied the stress resistance of Nile Tilapia fed diets containing DDGS and yeast, and also did not observed an improvement of survival and stress resistance. In other study conducted with the same species, Welker *et al*. (2014b) evaluated the partial replacement of soybean meal combined with corn meal by 30% of different sources of DDGS (sorghum, wheat, corn and whiskey) and they did not find differences in hematological or immune parameters.

Therefore, the present study was conducted to investigate if the dietary increasing levels of SDDGS may affect the immune and diseases resistance of Nile Tilapia to bacterial challenge.

Before the bacterial challenge the averaged hematological parameters observed in present study remained within the reference range established for Nile Tilapia (as well as to values determined in similar experimental conditions (Araujo *et al*., 2011, Hrubec e Smith, 2010, Ferrari *et al*., 2004; Tachibana *et al*., 2010; Barros *et al*., 2015). Before the bacterial challenge, the partial replacement of soybean meal by SDDGS did not affect the hematological and immune profiles. Interesting, even the highest replacement level of soybean meal by SDDGS, up to 70%, lead to no negative effect on hematological and immune innate parameters. Besides, even though not statistically significant, the total number of leucocytes of fish fed the 30% SDDGS diet was higher than that that fed with the other diets, while those fed the 10% SDDGS diet registered higher plasma lysozyme activity than with the other diets.

In the present study, challenge with *A. hydrophila* has been successfully performed for a Nile Tilapia, as 50-30% of the fish died after the bacterial infection. Abdel-Tawwab *et al*. (2008) reported that an intraperitoneal injection of *A.hydrophila* with a concentration of $5x10^5$ CFU ml^{-1} killed Nile Tilapia within 12 h and produced severe focal lesions. In the present study, all dead fish showed typical symptoms of *A.hydrophila* infection including marked hemorrhaging on the body surface, base of fins and the operculum. A study performed in the same experimental system, challenged Nile Tilapia with an intraperitoneal injection of 100 µL of $1.0x10^5$ CFU ml^{-1} *A.hydrophila* culture reported an mean survival rate of 70% (Barros *et al*., 2015).

Tilapia infected with *Aeromonas hydrophila* normally present lower hematological values. However, in the present study comparing the hematological and humoral immune parameters, before and after challenge, it was not possible to detected significant signs of inflammation due to infection by this bacteria, which are generally characterized by the increased of the number of neutrophils and monocytes in the blood and a reduction in the number of thrombocytes and lymphocytes, linked to the migration of these cells to the inflammation focus (Abdel-Tawwab., 2012; Barros *et al*., 2014; Iwashita *et al*., 2015). At least in part, these differences may be attributed to the time-lag between the pre and post-challenged blood sample collection, as in this study blood collection after challenged occurred after 15 days, when the infection and inflammatory response may not be in its pick.

Regarding the effect of the dietary inclusion of SDDGS, it was observed that the average number of days at which the first mortality occurred, following *Aeromonas hydrophila* challenge, and survival rate were higher in fish fed the 30% and 50% SDDGS diets than with the other diets. Also for the dietary inclusion of 30% SDDGS, it was observed an increase of the total neutrophils count before and after the bacterial challenge of about 52% and 29%, respectively, relatively to the non-SDDGS supplemented diet. Neutrophils are key components of the innate immune response, being one of the first cells to respond to inflammation. Its increase in fish fed SDDGS based diets may be indicative of a potentiated systemic response, which may provide a more rapid non-specific immune response against the pathogenic infection (Standen *et al*., 2013). Moreover, the absolute and relative proportion of thrombocytes increased in all SDDGS based treatments relative to control diet. This increase was higher in fish fed the 30% SDDGS diet where Trb fold change values doubled relative to control values (increased about 113%, relatively to the control diet). Higher number of thrombocytes in SDDGS based diets relatively to the SDDGS non-supplemented diet may suggested a

reduction of the migration of thrombocytes to the inflammation focus, as previously reported (Lamas *et al*., 1994; Garcia *et al*., 2007).

Total serum protein is often used as an indicator of physiological condition in fish, as it is one of the most stable components of blood, and so an increase or decrease of total blood proteins, globulins and albumin has clinical relevance in fish (Peres *et al*., 2015). Infection may be followed by marked changes on total blood protein due to impair hepatic synthesis of blood protein, increase catabolism or losses of albumin in urine or synthesis of globulins by the immune system. High plasma albumin and/or globulin has been related to stress, inflammatory and innate immune responses or to feeding immunostimulants (Peres *et al*., 2015). Also higher levels of plasma non-specific humoral immune parameter, such as lysozyme and complement activity, have been used as indicative of immuno-enhancing properties to certain dietary compounds. In the present study, plasma albumin and albumin/ globulin ratio levels post-challenge also increased in fish fed the 30% SDDGS diet (23 and 69%, respectively) and 50% SDDGS (56 and 30%, respectively) relative to control diet (Figure 18). Similarly, the highest lysozyme activity (7.1 µg ml-1), corresponding to an increase of about 29% relatively to the control diet, was observed in fish fed the 30% SDDGS diet. However, complement activity was not affected by different dietary inclusion of SDDGS, suggesting an action of SDDGS inclusion

Even though no significant difference were observed, in present study, dietary incorporation of SDDGS seems to have some immunostimulating proprieties. Indeed, mortality of Nile tilapia exposed to *Aeromonas hydrophila* was lower with the 30 and 50% SDDGS diets. Also total number of neutrophils, plasma albumin, albumin/globulin ratio and lysozyme activity were increased with the SDDGS incorporation. Differences among these results and the aforementioned, obtained by other authors, may be related to the different source of DDGS (sorghum, corn or wheat) and technical process, species specific differences, such as the tolerance to the dietary fiber content and DDGS inclusion levels. Also the yeast percentage of DDGS, which greatly vary depending on the manufacture may also have some effect on the potential immune modulation of the DDGS.

Conclusion

Together, these results suggest that the replacement of SBM by 30 to-50% of SDDGS may enhance the fish resistance against bacteria and may up-regulate innate immune mechanisms. Additionally, and in contrast with other dietary treatments, the SDDGS 50% diet was the only treatment where no fish died in first five days of bacterial challenge. Besides, even the highest replacement level of SBM by SDDGS did not compromise the bacterial resistance of Nile tilapia. Further research, are need to corroborate the trend observed in this trial, including supplementary immunological tests, as well as a full characterization of the SDDGS use mainly in which concern the yeast fraction of the SDDGS. Present results are very promising and may give and new add-value to an agro-industrial by-product with low economic value.

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